Photography THROUGH THE Microscope

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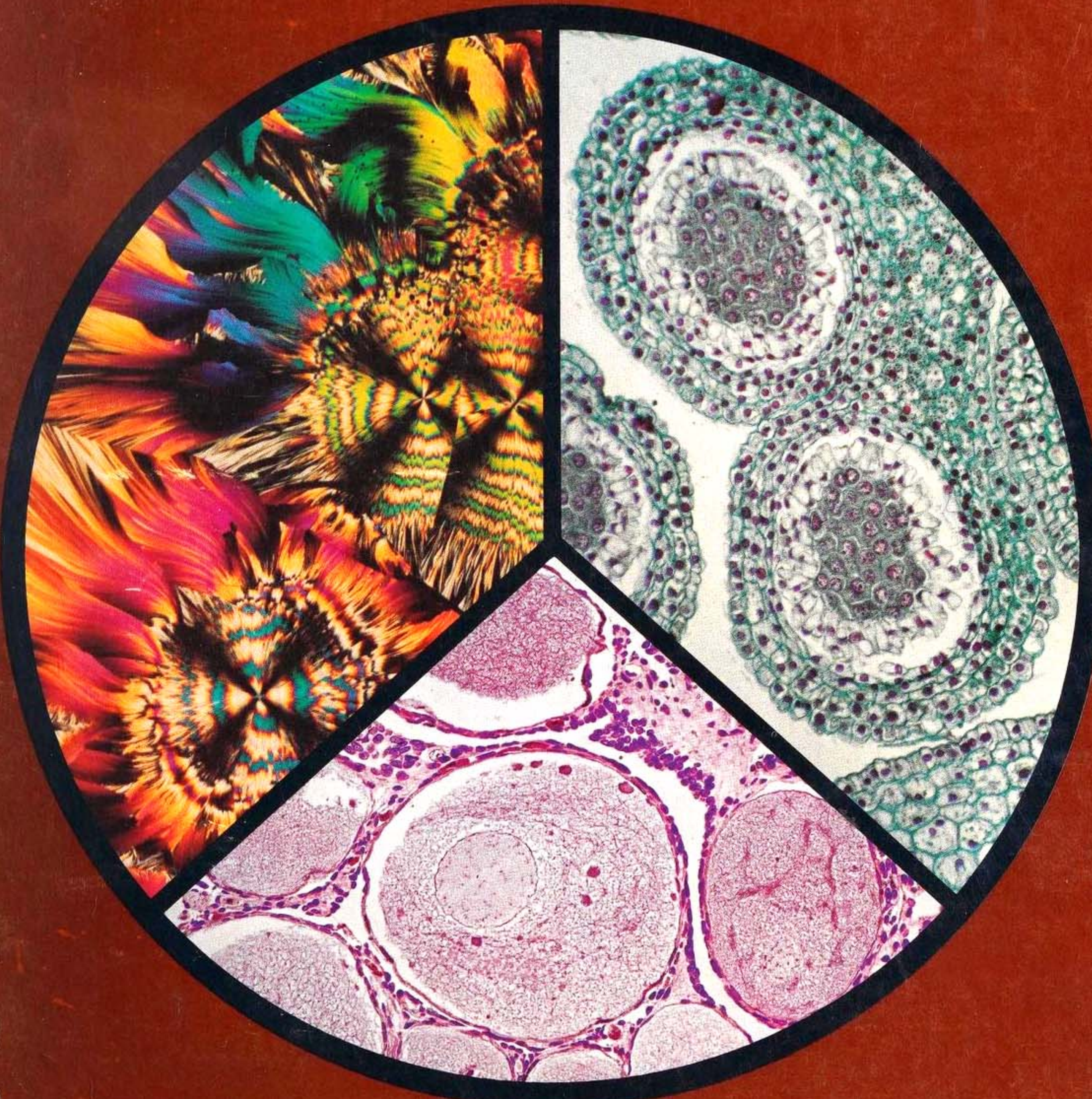
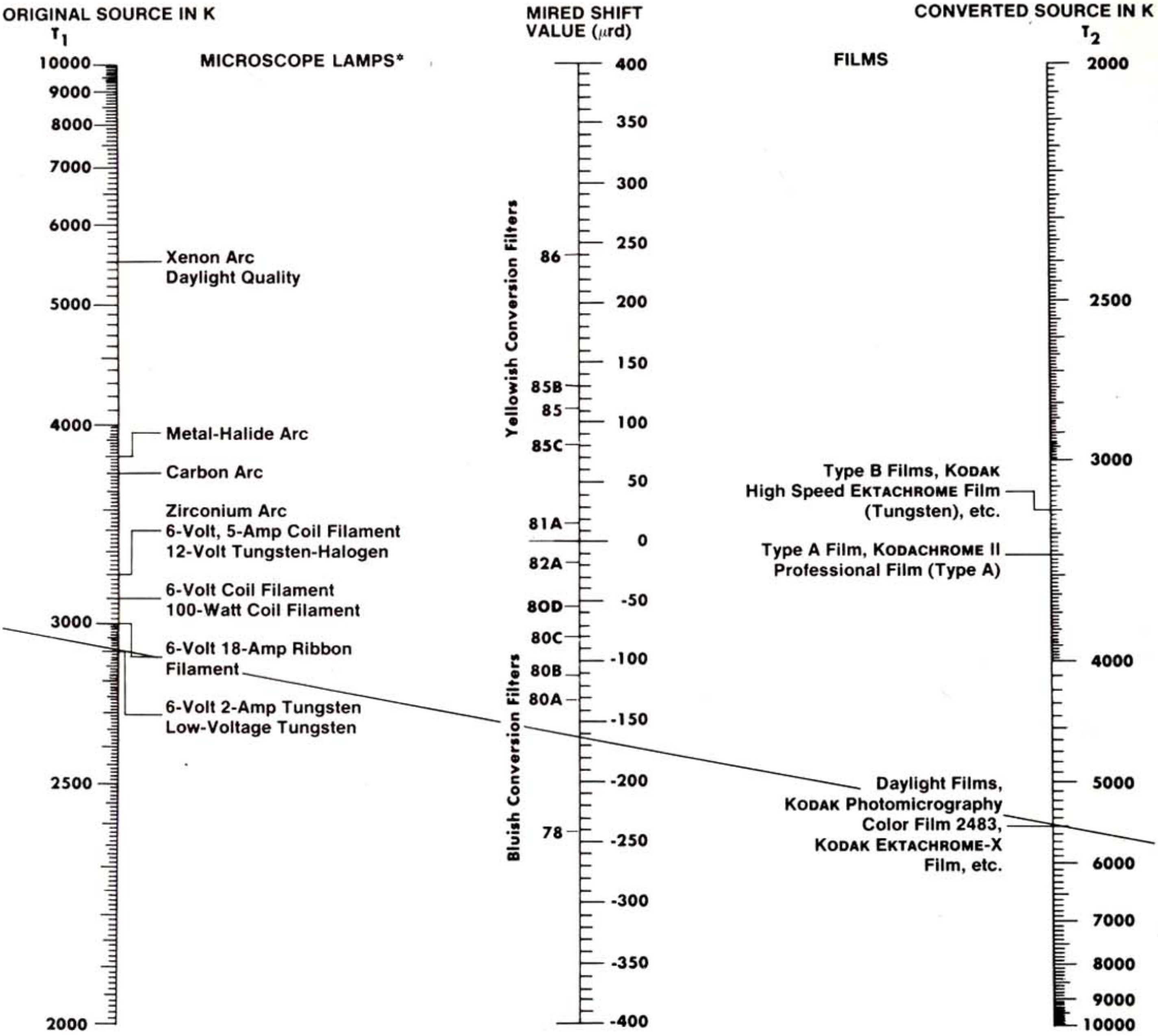


MIRED NOMOGRAPH FOR LIGHT SOURCE CONVERSION



The Mired Nomograph can be used to find the filter required for a particular conversion by placing a straightedge from an original source (T_1) to a second source (T_2) as illustrated above by the diagonal line. In the illustration, tungsten illumination at 2900 K requires an approximate − 163 mired shift to convert to daylight illumination at 5500 K. Kodak Wratten Filter No. 80A + 82B with a mired shift value of − 163 meets this requirement.

*Many microscope illuminators are rated at 2700 to 3200 K depending upon the voltage being used. However, these values are approximate and should be used only as a guide. For critical work a practical test should be run. Consult your microscope dealer for lamp information.

COVER PHOTOGRAPH:

LEFT: Hippuric crystals with polarized lighting— × 150.

Center: Ovary of amphiuma— × 100. Hematoxylin and eosin stains. Kodak Ektachrome Film 6116, Type B (Process E-3). A didymium filter enhanced the eosin stain color.

Right: Cross section of lily bud— × 200. Kodak Ektacolor Professional Film, Type S.

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Please Note: Since the last edition of *Photography Through the Microscope* was published, several significant developments in Kodak films for photomicrography have occurred. In this edition you will find applicable technical information on KODAK Photomicrography Color Film 2483 and KODAK Photomicrography Monochrome Film (ESTAR-AH Base) SO-410. Complete technical information regarding processing, physical properties, and sensitometric data is available on request from Department 412-L, Eastman Kodak Company, Rochester, New York 14650. Ask for Publications No. P-302 (for 2483 Film) and No. P-304 (for SO-410 Film).

Punched to fit the **Binder for Kodak Technical Information** (W-4), sold by photo dealers.

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INTRODUCTION

The technique of making photographs by means of a compound microscope is called *photomicrography*. It involves the coupling of a camera to a microscope and the efficient use of illumination. The quality of a "photomicrograph" depends almost entirely on the quality of the image produced in the microscope. A complete understanding of the principles and operation of the microscope is therefore the first prerequisite to producing good photomicrographs.

Photomicrography involves the production of large recorded images of very small, microscopic objects. Photomicrography should not be confused with *microphotography,* which involves making extremely small photographic images of large objects.

Although any type of microscope can be used to make photomicrographs, the most common is the "brightfield" illuminated microscope. The specimen, or subject, is either dark or colored and appears against a bright, almost white, background. Hence the term "brightfield." This microscope is found in all biological laboratories and in all schools where biology is taught.

In order to use a microscope efficiently, you must have a good working knowledge of the instrument. This knowledge includes the capabilities and limitations of the optical components, how the microscope is adjusted, how the illumination of the specimen is applied, and even how specimens are prepared for examination. If you intend to make photomicrographs, you should have some knowledge of photography, including an understanding of sensitized materials and how a camera can be suitably attached to a microscope.

Photomicrographs are made for a variety of reasons, but the principal one is for teaching. It is much easier to project a color slide for class viewing than to have each member peer into the microscope. A photomicrograph is often used as a research record, or to illustrate a particular phenomenon or condition in a published article. Photomicrographs of metals are very often made to supplement engineering or metallurgical reports.

Photomicrography is used in practically every field in which a microscope is the fundamental tool—whenever an enlarged, recorded image would prove useful. Photomicrography is important to teachers and students in high schools and colleges for laboratory studies in biology, botany, zoology, anatomy, etc. It is probably of even greater importance in industry, medical research, pathology, criminal investigation, agriculture, and forestry.

Whatever the purpose of a photomicrograph, you should remember that the recorded image is no better than the image produced in the microscope, except that contrast can be enhanced photographically. Thus, the first step in photomicrography is to understand the compound microscope and how to use it to best advantage in visual applications.

UNDERSTANDING THE COMPOUND MICROSCOPE

GENERAL PRINCIPLES

A "simple" magnifying system uses a single lens unit to form an enlarged image of an object for viewing or for projecting. An example of the latter is slide projection where a transilluminated transparency is enlarged to form a real image on the screen. If the screen were removed and a suitable lens located behind it and focused in the plane of the screen, the lens would form another image of the slide. This is the basic principle of a "compound" magnifying system—the one found in a compound microscope. The observer looks at the first, or primary, image with a lens that produces an enlarged secondary image, called a virtual image. That is the image the eye perceives. (See Figure 1.) Another feature of microscopes is that the lenses are of relatively short focal lengths. The shorter the focal length, the greater the magnification at a given image distance. In a microscope a high, two-stage enlargement is attained over a relatively short optical path with such short focus lenses.

The first lens in a microscope is called the *objective,* since it is near the object. This lens projects a magnified image to a fixed position. The amount of magnification that the objective produces at this fixed distance is called its "magnifying power." The magnifying power of an objective is classified as 5×, 10×, 20×—up to 100×. The projection of the primary

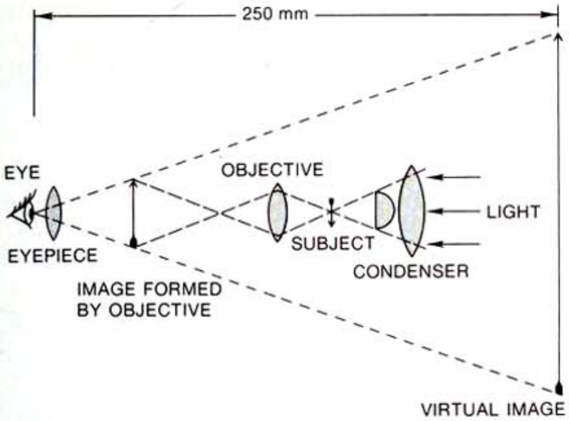

FIGURE 1—Simplified Diagram—Principle of compound microscope. The intermediate image formed by the objective is enlarged by the eyepiece. The virtual image is seen by the eye. Alternatively, the eyepiece can project a real image into a camera.

image takes place within the body tube of the microscope; the distance from the back focal plane of the objective to the primary image is called the "optical tube length."

The second lens is placed in the body tube above the primary image. This lens is called the "eyepiece," and forms a secondary, further enlarged image within the microscope. The eyepiece (often called the "ocular"), like the objective, is also classified in terms of magnifying power, and could be 5×, 10×, or higher—up to 25×.

The total amount of image enlargement, or "magnification," produced within the microscope is found by multiplying the magnifying power of the objective by that of the eyepiece. A 10× objective and a 10× eyepiece then produce a "visual" magnification of 100×, or ×100 as it is commonly written.

As light rays emerge from the eyepiece they converge to a point called the "eyepoint." This is the position which the eye normally seeks when you look into a microscope to see the whole image field in the microscope. The eyepoint is also often referred to as the "Ramsden disk" or "Ramsden circle." The distance from the eyepoint to the virtual image, or final image, within the microscope system is 250 mm (10 inches).

Instead of looking into the microscope, you can allow the image to be projected from the eyepiece to the film plane. If the film plane, or camera, is 250 mm from the eyepoint, the magnification at the film plane will be the product of the eyepiece and objective magnifications.

The very fine details in a micro-specimen must be distinguished by the objective lens. This lens must be of high enough quality to "resolve" these details and produce an efficient primary image. The main purpose of the eyepiece is to further enlarge the image formed by the objective. This image can be degraded by an eyepiece of low quality, but it cannot be improved (as far as image resolution is concerned), regardless of eyepiece quality.

OBJECTIVE

Types of Objectives

Microscope objectives are usually classified in terms of magnifying power, mechanical tube length, numerical aperture, and their degree of optical correction, that is, whether they are achromats, apochromats, or fluorites. Some of this information and the focal length is usually imprinted on the objective mount.

The *achromat* is the most common type of objective used on any microscope. It is also the least expensive. An achromat is corrected for spherical aberration for one color only, usually yellow-green. It is corrected for chromatic aberration for two colors. If an achromat is used with white light, color fringes may appear in the outer margins of the image. When black-and-white film is used, these fringes may contribute toward a fuzzy image. If light of one general color (such as green) is used, the image will be much sharper. When monochromatic green light is used—that is, light of a single wavelength or a narrow band of wavelengths—the image will be much better. Therefore, the best image will be obtained in photomicrography when a green filter is placed in the light beam. Images of inferior quality may result when light of longer or shorter wavelength is used.

An *apochromat* represents the finest microscope objective available. It is corrected for spherical aberration for two colors (blue and green) and for chromatic aberration for the primary spectral colors of red, green, and blue. Because of this high degree of correction for aberrations, the apochromat is particularly suitable for color photomicrography and for the best resolution of fine details and the finest image quality. Apochromats, however, achieve their finest correction when used with matched compensating eyepieces.

The third type of objective is known as a *fluorite,* or "semiapochromat." Objectives of this type are better than achromats, but not quite as good as apochromats. They represent a compromise, both in quality and in cost. Like the apochromats, they should be used with compensating eyepieces for best performance.

All types of objectives mentioned above exhibit a certain amount of "curvature of field." The image may be sharp in the center of the field, but sharpness

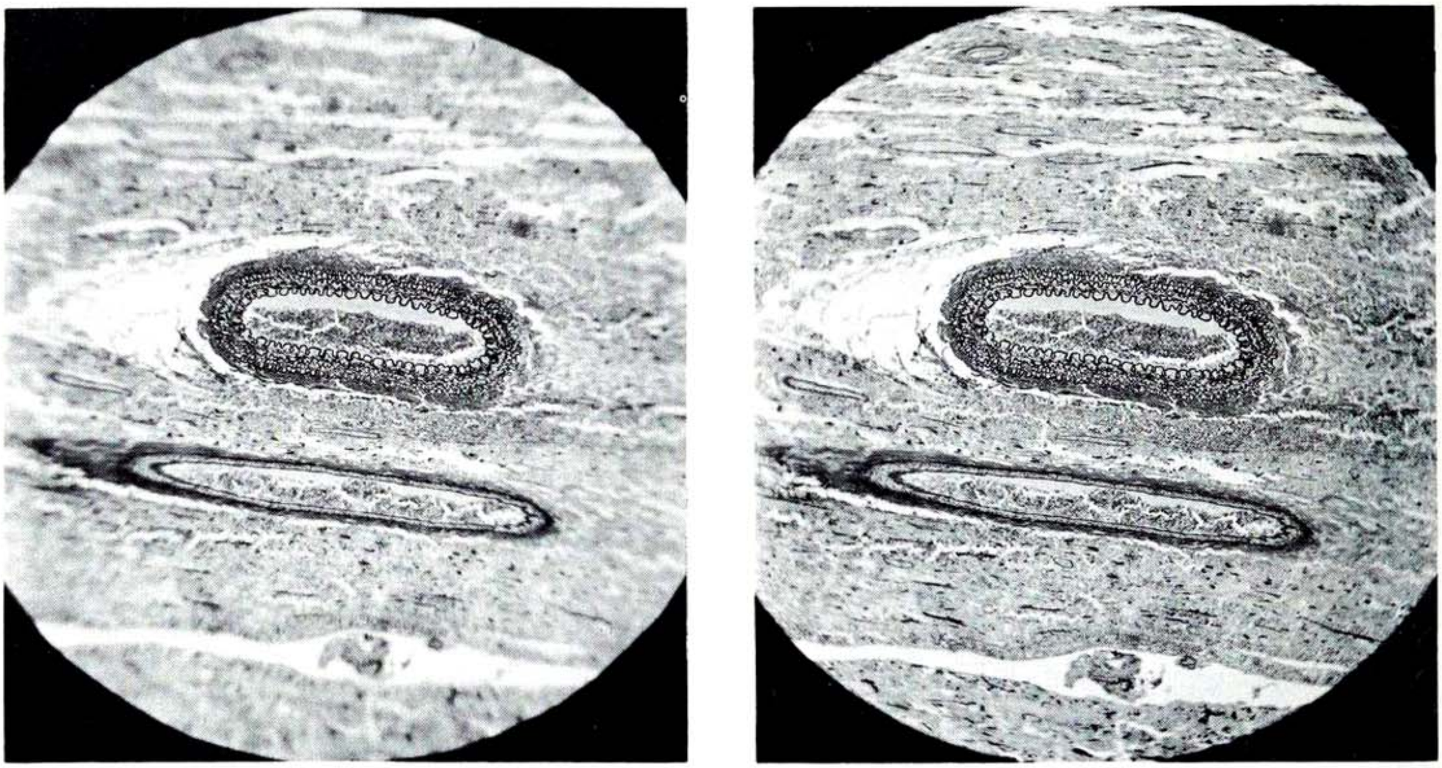

FIGURE 2—Elastic fibers in human artery, × 40, (a) Curvature of field; (b) Corrected curvature of field.

falls off toward the periphery. This effect can be overcome to some extent in photomicrography by limiting the recorded area to the center of the field, when possible. It can also be reduced appreciably by using a special eyepiece, described further on.

However, it is better to use "flat-field" objectives, made in the above types. Their names are preceded by a prefix, "plano" or "plan"—such as plano-achromat, plan-achromat, plano-apochromat, or plan-apochromat. Curvature of field has been corrected in the optical design of these objectives, and they are particularly suitable for examination or photomicrography of large fields.

"Flat-field" objectives are used in conjunction with compensating eyepieces or eyepieces designated specifically for them. Focus is then relatively uniform from the center to the periphery of the field. (See Figure 2.)

Most objectives intended for use with transmitted light must be used with "covered" objects. That is, a "cover glass" must be mounted over the specimen. The thickness of the cover glass is specified as either 0.17 mm or 0.18 mm, depending on the objective. The specification for cover-glass thickness results from the fact that objectives are corrected for spherical aberration only when used with a cover of the right thickness. Deviation from this thickness can result in appreciable overcorrection or undercorrection for spherical aberration, particularly with high-aperture, dry objectives.

Objectives intended for use with reflected light require "uncovered" objects. No cover glass is used over the specimen. This principle applies mainly to metallurgical microscopes and metal specimens.

Most of the objectives used in a brightfield microscope are considered "dry." This means that only air exists between the front of the objective and the specimen slide. Some objectives are of the "immersion" type; a liquid medium, usually a special oil, is placed between the objective and the specimen slide.

Tube Length

The objective is screwed into the bottom part of the microscope body-tube, while the eyepiece is inserted into the top "draw-tube" of the microscope. The distance between the insertion position for the objective and the top of the draw-tube is called the "mechanical tube length."

Some manufacturers specify a mechanical tube length of 160 mm, whereas others specify 170 mm. Objectives are usually designed optically for a specific tube length. If they are used at a different tube length, they will not retain their intended optical efficiency. They should not, therefore, be interchanged on different microscopes if the specifications are not known. Image quality may suffer if this practice is followed. Some microscopes are equipped with adjustable draw-tubes, so that mechanical tube length can be altered to comply with the specification for an objective. These microscopes are rare, however. Most modern microscopes have permanent tube lengths designed by the manufacturers of their optics. The best practice for a novice is to use objectives of one make only, on a microscope of the same manufacture.

Some microscopes employ objectives with "infinity" correction. Mechanical tube length is not fixed. Such objectives should *never* be used on a microscope of other manufacture, nor should other objectives be used on a microscope designed for infinity-corrected optics. Image quality will be very poor in either case.

When an objective is used at other than its specified tube length, its magnifying power will be different than indicated. For example, a 10× objective designed for 160 mm tube length will magnify more than 10× if used at a 170 mm or longer tube length; conversely, magnifying power will be reduced at lengths shorter than the intended tube length.

In addition to erroneous magnifying power, poor image quality will also result if correct tube length is not used. Too long a tube length will result in overcorrection and too short a tube length will result in undercorrection; both will result in poor definition (image sharpness).

Optical Aberrations

If a simple, positive lens were used to project the primary enlarged image, the quality of the image would be very poor due to inherent optical aberrations. Aberration is a failure of a lens to produce exact point-to-point correspondence between an object and its image. In order to improve image quality it is necessary to design lenses so that this aberration is corrected as much as possible. Microscope objectives are corrected for spherical and chromatic aberrations.

Spherical aberration occurs when light rays passing through the central and outer portions of a lens are not brought to focus at the same distance from the lens. This condition arises because light is refracted more at the edge of the lens with gradual reduction to zero at the optical center. The image of a point is not reproduced as a point but as a larger, circular area. The image of an object that is composed of an infinite number of points cannot possibly be sharp as long as spherical aberration is present. Also, white light is composed of all colors—which are refracted and focused differently, depending on specific wavelength. It is possible to correct spherical aberration for one color, but it might not be corrected for another color. This aberration can be partially corrected, however, by formation of an image at a fixed distance; thus the specification of a mechanical tube length. See Figure 3 for a diagram.

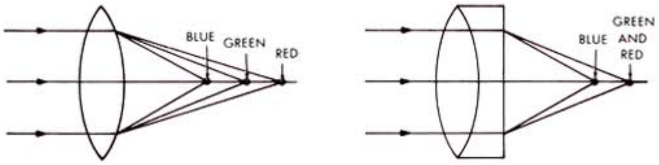

FIGURE 3—Left, a simple, uncorrected lens focuses three primary colors at different points on the optical axis. Right, an achromatic lens will focus two primary colors, usually green and red, at the same point on the optical axis.

Chromatic aberration occurs because the focal length of a simple lens varies noticeably with wavelength. Blue rays are shorter in wavelength and focus closer to the lens than green or red rays. The lens is unable to bring light of all colors to a common focus. This effect can be corrected to a practical degree in the design of a microscope objective. The amount of correction is specified for each type of objective.

The sharpness of the image produced in a microscope is limited by the degree of correction for spherical aberration. If chromatic aberration is present in the microscope objective, color fringes will be evident in the image.

Numerical Aperture

In conventional photography, photographic lenses are always classified in terms of *f*-value, which is an indication of light-gathering power. A "fast" lens might be *f*/1.4 or *f*/2.0. Slower lenses might have *f*-values such as *f*/4.5, *f*/5.6, or *f*/8. The *f*-value of a photographic lens is determined by dividing the focal length by the apparent diameter of the diaphragm opening.

Microscope objectives, however, are not classified in terms of *f*-value. We are not concerned in microscopy with light-gathering power, but with the ability of the lens to distinguish fine structural details in a specimen. This is the principal reason for obtaining a magnified image—to see the details within the specimen. This ability is expressed in terms of *numerical aperture,* or "N.A." as it is usually called.

Mathematically, numerical aperture is expressed as the product of the refractive index (n) for the medium in which the lens operates and the sine of one-half the angular aperture of the lens (u). The formula then reads: N.A. = n sine u. The sine relationship is a simple means for expressing the size of an angle as a number, the ratio of two sides of a right triangle.

The numerical aperture for any objective is *always* imprinted on its mount. A 10× achromatic objective usually has an N.A. of 0.25, and a 20× achromat will usually have an N.A. of 0.50. Apochromatic objectives have higher numerical apertures than achromats of similar magnification.

Some objectives are to be used "dry"; air is the medium between the front of the lens and the specimen slide. Air has a refractive index of 1.00, so that the formula for numerical aperture for a dry lens will read: "N.A. = 1.0 times sine u" or just "sine u." The largest numerical aperture for a dry lens is about 0.95; it *never* reaches 1.00.

In order to have an objective of numerical aperture equal to or greater than 1.00, a liquid medium must be placed between the lens and the specimen slide. An "immersion oil" is often used for this purpose; its refractive index is usually about 1.51. Objectives intended for immersion always have an indication on the lens mount. There are also special immersion lenses, which require distilled water (n = 1.30) or glycerin (n = 1.48). These are rare, however, and are not generally used with brightfield microscopes. The numerical aperture for oil-immersion objectives ranges from 1.00 to 1.40.

Working Distance of Objectives

The distance between the front lens of an objective and the top surface of the cover glass on a specimen slide is called the "working distance" for the objective. Objectives are designed optically for use with a cover glass of specific thickness, usually either 0.17 mm or 0.18 mm, depending on manufacture. Any deviation from the specified thickness when a dry objective is used will introduce spherical aberration, which may affect image sharpness. A cover glass that is too thick will cause *overcorrection* for spherical aberration. Conversely, too thin a cover glass introduces *undercorrection.* The effect is most noticeable with high-aperture, dry objectives.

Working distance is directly related to the allowable movement of the objective in obtaining critical focus of the specimen image. It decreases rapidly as the focal length of the objective decreases; for oil-immersion objectives, the working distance is measured in fractional parts of a millimeter. Cover-glass thickness is of lesser importance as far as introducing spherical aberration is concerned, since the oil has about the same refractive index as the cover glass. The allowable movement of an immersion objective, however, *is* affected by cover-glass thickness. More working distance is obtained when the cover glass is thinner than specified or when *no* cover glass is used, as is often the case with blood smears. A thicker cover glass will *decrease* the working distance; the thickness can then approach the point where focus of the specimen may become impossible. Other minor factors which can affect the allowable working distance for an immersion objective are the viscosity of the oil and the amount of mounting medium between the cover glass and the specimen. The effects of these factors are small, but when they are added to the cover-glass thickness, they contribute toward a reduction in working distance.

The following table shows the average working distances for different objectives of various focal lengths. The figures can only be considered approximate, since objectives of different manufacture will differ in characteristics. They are, however, fairly typical.

TABLE I

Numerical Aperture (N.A.) vs Working Distance

Objective Type	N.A.	Focal Length (mm)	Working Distance (mm)
Achromat 10×	0.25	16	7.70
Apochromat 10×	0.30	16	4.85
Achromat 20×	0.50	8	1.60
Apochromat 20×	0.65	8.3	0.50
Achromat 45×	0.85	4	0.30
Apochromat 47.5×	0.95	4	0.18
Achromat 97× oil	1.25	1.8	0.13
Apochromat 90× oil	1.30	2.0	0.12

Resolving Power

The ability of the optical system in a compound microscope to distinguish and separate fine structural details in a specimen is known as "resolving power."* It is limited by the N.A. of the objective, but it also depends upon the working N.A. of the substage condenser. The higher the N.A. of the system, the greater will be the resolving power. Resolving power is also dependent on the wavelength of light. The shorter the wavelength, the better the resolving power. The process of making fine details visible is called "resolution," and is usually expressed in terms of micrometers, formerly called microns. (One micrometer, μm, equals 0.001 millimeter.)

The formula for computing resolving power is $R = \frac{\lambda}{2\ N.A.}$ where R is resolving power, in micrometers; λ is wavelength, also in micrometers; and $N.A.$ is, of course, numerical aperture. When the optical system is correctly aligned and adjusted, the N.A. of the objective can be used in the formula.

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*However, this value is subjective. An image may be unsharp but may still be considered resolved.

With regard to wavelength, the finest resolving power is obtained with ultraviolet radiation, which represents the shortest usable wavelengths. In the visible region of the spectrum, blue light has the next shortest wavelength, then green, and then red. If white light is used, the applicable wavelength is that for green—the middle of the visible spectrum and region of highest visual acuity.

If the dominant wavelength for green light (.550 micrometer) and an achromat of high N.A. (1.25) are applied in the formula, the resultant resolving power is 0.22 micrometer. With green light, then, it is possible to resolve particles as small as 0.22 micrometer (0.00022 mm) using an achromat. The following table shows the effective resolving power with several achromatic objectives of different numerical apertures when green light is used.

Resolving Power for Achromats

Magnifying Power	4×	10×	20×	45×	100× oil
Numerical Aperture	0.10	0.25	0.50	0.85	1.25*
Resolution (micrometers)	2.75	1.10	0.55	0.32	0.22

*Highest N.A. for an achromat.

The resolving powers for apochromatic objectives are better than those for comparable achromats, since they are more highly corrected optically and have higher numerical apertures. The following table shows pertinent, comparable data for apochromats, again with green light. (Apochromats lower than 10× usually are not manufactured.)

Resolving Power for Apochromats

Magnifying Power	10×	20×	47.5×	90× oil
Numerical Aperture	0.30	0.60	0.95	1.40*
Resolution (micrometers)	0.92	0.46	0.29	0.19

*Highest N.A. for an apochromat.

Resolving power for an apochromat of the highest N.A. (1.40) with light of different wavelengths is shown in the following table. This indicates the improvement of resolution with shorter wavelengths of light.

Change in Resolution with Wavelength

	Green	Blue	Ultraviolet
Wavelength (micrometers)	.546	.436	.365
Resolution (micrometers)	0.19	0.16	0.13

Therefore, it is possible to resolve particles as small as 0.13 micrometer (0.00013 mm) by using ultraviolet radiation.

Although it is not expected that everyone will need to determine the exact resolving power for any objective, it is important to understand the capabilities and limitations of a lens in order to use it to best advantage. This knowledge should help in the selection of a lens or of the type of light needed to photograph a specimen most efficiently.

EYEPIECES

The purpose of the eyepiece in a compound microscope is to enlarge the primary image formed by the objective, and to either render it visible as a "virtual" image in the microscope or project it as a "real" image that can be recorded in a camera.

For visual examination of the image in the microscope, any type of positive eyepiece can be used satisfactorily. The most common eyepieces are called "Huygenian" and "wide field." Although Huygenian eyepieces can be used with low- and medium-power achromatic objectives in photomicrography, color fringing and poor image quality may result if they are employed to make high-power photomicrographs. The principal advantage of "wide field" is in visual work, to scan a slide in order to find a suitable field for photography.

Whenever an apochromatic objective is to be utilized for photomicrography, the eyepiece should be of the "compensating" type in order to provide the high image quality and flat field of which the objective is capable. This is especially true of plan- or plano-objectives. Apochromats inherently produce what is called "chromatic difference in magnification." Compensating eyepieces are overcorrected for this effect and will produce images free from color defects. This type of eyepiece can also be used effectively with semi-apochromats and the high-power achromats.

Eyepieces designated as "Hyperplane" and "Periplan" are excellent for black-and-white photomicrography, particularly with filtered light. They will provide reasonably flat fields, thus overcoming the effect of "curvature of field," which is inherent in image-formation by most objectives.

Some eyepieces are intended specifically for photomicrography; they are, appropriately, called "photo eyepieces." Besides producing fair flat-field results, they are also color-corrected and, therefore, of advantage in color photomicrography.

Eyepieces, generally, are produced with different magnifying powers, ranging from about 4× to 25×. The most common are those with a magnifying power of 10× or 15×.

CONDENSER

The third optical component of a compound microscope is called the "substage condenser." The specimen slide is placed on a platform beneath the objective. This platform is called the "stage"; the substage area is beneath the stage. In some microscopes, light is reflected from a mirror, and through the substage condenser to illuminate the specimen. Many microscopes today have built-in substage illumination, so a separate mirror is not used. In either case, however, the light is directed through the substage condenser and converges to a very small area at the position of the specimen. The light rays diverge as they pass through the specimen and form an inverted cone, whose base is just large enough to fill the aperture of the objective. The size of the light beam entering the condenser is controlled by the opening of a variable diaphragm beneath the condenser. This is called the "aperture diaphragm." Both the focus of the condenser and the opening of the diaphragm are adjusted for Kohler illumination. (See page 29.)

Three types of substage condensers are available. The Abbé condenser is the simplest and least expensive type. It is provided on most microscopes unless another type is specified. This condenser is *uncorrected* for optical aberrations, and may have either two or three lens elements. The top element can sometimes be removed, either by unscrewing it or by swinging it away on a pivot, for low-power work (10× objective or lower). For medium- and high-power work, it remains in place. If the top element is not removed, it may be impossible to illuminate the entire field seen by the low-power objective. Consult the section, "Chromatic Aberration," on page 43 for a discussion of condenser adjustments to obtain good color balance.

The second type of condenser is called "aplanatic," and is optically corrected for spherical aberration. This type is *not* available from all manufacturers, but is of better quality than the Abbé condenser.

The "achromatic" condenser is the best available and is corrected optically for both spherical and chromatic aberrations. It is highly recommended for photomicrography, particularly with color film. Both the aplanatic and the achromatic condensers are divisible, like the Abbé condenser, for use in low-, medium-, and high-power work.

Any substage condenser should have centering screws so that it can be correctly aligned with respect to the objective. This is an important feature in photomicrography and should definitely be considered when a microscope is purchased.

The numerical aperture of a complete substage condenser should always be, and usually is, equal to or greater than the largest numerical aperture of the available objectives. The opening of the substage diaphragm can then be adjusted to match the working N.A. to that of the objective in use.

When an objective lens is intended for oil immersion and its numerical aperture exceeds 1.00, immersion oil of the same type should be placed between the microscope slide and the top lens of the condenser. If this step is omitted in high-power photomicrography, the numerical aperture of the condenser will be limited to 1.00, the refractive index of air. Because the effective numerical aperture of the objective is also limited to 1.00, the resolving power of the system will be lower than when oil is placed on the condenser.

ACCESSORIES

Mechanical Stage

The mechanical stage is one of the most useful accessories for a compound microscope. The stage is a device both for holding a specimen slide firmly in position and for moving it smoothly, either back and forth or right and left. This feature is of particular importance, since it enables the user to scan a slide easily in order to locate a suitable field for photography. Then, if the mechanical stage is graduated, it becomes possible to note the graduation figures for the position of a specific field for future reference, in case the same field must be relocated and, possibly, rephotographed.

A mechanical stage is not always included with a microscope, so it is necessary for the buyer to specify that one *be included.* If a microscope in use does not include this type of stage, it is often possible to purchase an attachable stage separately.

A rotating, mechanical stage is the most useful of all stage types. The rotating stage enables the user to adjust image composition to better suit the rectangular format of the film frame. However, if a rotating stage is not available and the camera over the microscope can be rotated, the same effect can be achieved.

Field Finder

In addition to a graduated mechanical stage, another device for accurately relocating fields on a specimen slide is called a "field finder." This is a microscope slide containing a grid with rectangular coordinates, which are numerically indentified.

When a particular field has been located on a specimen slide, the slide is carefully removed from the mechanical stage and the field-finder slide is put in its place. The coordinates for the visible field are then recorded for future reference. Then, whenever the original field must be relocated, the field-finder slide is placed on the stage and the stage is adjusted to the recorded coordinates. The specimen slide is then substituted for the field finder, and the original field is visible.

If a graduated mechanical stage is used to locate and relocate fields, the technique is satisfactory as long as the same microscope or one with an identical stage is used. Graduated stages, however, vary from one manufacturer to another, and often between microscopes of the same manufacture. The field-finder slide provides the advantage of being interchangeable between microscopes.

Field-finder slides can be purchased either from biological supply firms or microscope manufacturers.

MAGNIFICATION

In photomicrography, image size (or magnification) is controlled by the magnifying powers of the objective and eyepiece, and by the bellows extension (or eyepiece-to-film distance). When you look into a microscope, the visual magnification is equal to the product of the magnifying powers of the objective and eyepiece. When the micro-image is projected from the eyepiece, this magnification is reproduced at a distance of 10 inches (250 mm) above the exit pupil of the eyepiece. If the distance is greater than 10 inches, magnification will be *increased* proportionately. If the distance is less than 10 inches, magnification will be *decreased* proportionately. For example, if the objective is designated as 20× and the eyepiece as 10×, visual magnification in the microscope will be ×200. Then, if the image is projected to a ground glass screen (or film plane) 20 inches above the eyepiece, magnification will be ×400. But, if the projection distance is only 5 inches, magnification will be only ×100, etc.

When the magnifying power of either the objective or the eyepiece is unknown, or when the exact eyepiece-to-film distance is unknown, it becomes necessary to measure magnification. This can be done by means of a calibrated scale called a "stage micrometer." Such a scale consists of finely ruled lines on a microscope slide, with a finite separation in decimal parts of either inches or millimeters. In use, the stage micrometer is placed on the microscope stage and its magnified image is projected to the ground glass or film plane of the camera. By measuring the separation of lines on the ground glass and comparing it with the original separation on the slide, image size can be measured directly. When no ground glass is used, the image of the lines can be recorded on film and measured there after the film is processed (Figure 4).

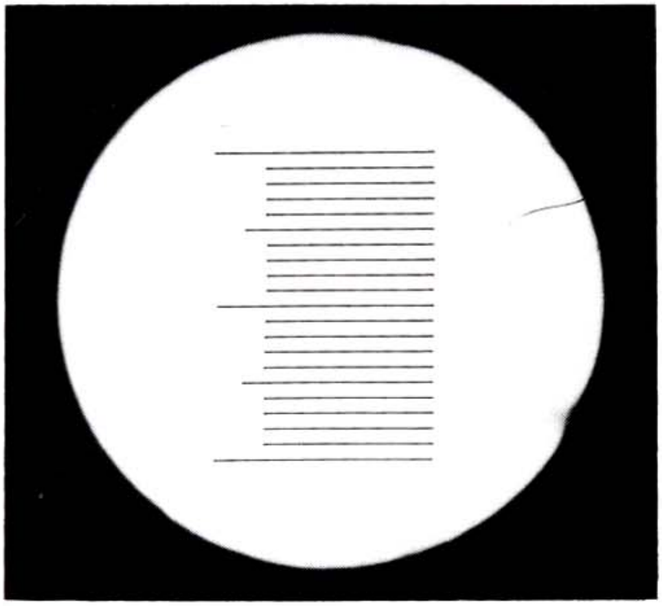


FIGURE 4—Magnified image of eyepiece micrometer disk.

It is sometimes desirable to record a micrometer-scale image simultaneously with a specimen image. This can be done in either of two ways. The stage micrometer image and the specimen image can be recorded together by double exposure: first one image is exposed, then the other, on the same film. In this case, it is advantageous to have a micrometer scale with white lines on a dark background. Another technique is to use a focusing eyepiece with a micrometer scale included. In this case, the micrometer scale in the eyepiece is calibrated with a stage micrometer beforehand so that the line separations will be known. Then, both eyepiece scale and specimen image are recorded in one exposure.

The usable limit of magnification that can be achieved with any combination of objective, ocular, and bellows draw is determined by the numerical aperture, or N.A., of the objective. Each objective has its numerical aperture engraved on the mount. An achromat of 6 mm focal length might have an N.A. of 0.65; a 4.3 mm fluorite, an N.A. of 1.00. N.A. is important in figuring the capability of objectives.

If magnification in the microscope is carried beyond the point of resolution—that is, no more detail is resolved—we have a condition called "empty magnification." The N.A. of an objective can be used to compute maximum usable magnification by a "rule of thumb." If the N.A. figure is multiplied by 1000, we will obtain a figure which, although not exact, is close enough for practical purposes. An N.A. of 0.65, then, will allow usable magnification up to ×650. If more magnification and better resolving power are

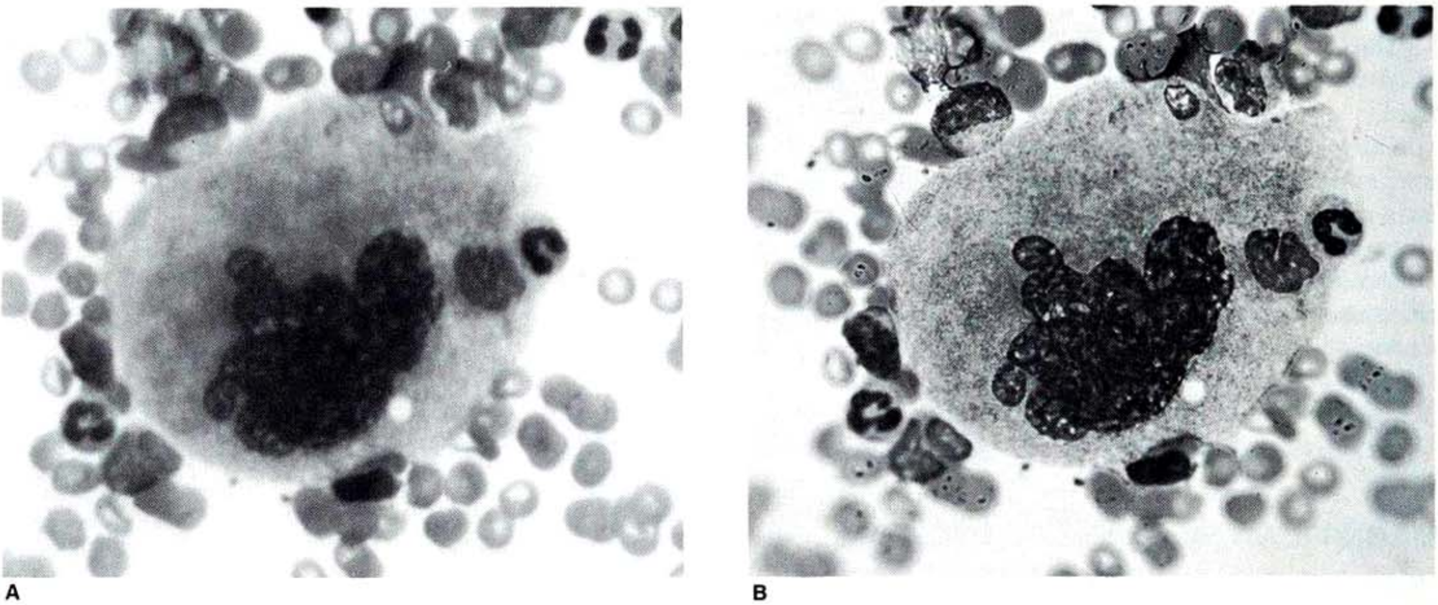


FIGURE 5—(a) Empty magnification because of insufficient resolving power of objective (16 mm achromat N.A. 0.25), ×500. (b) Significant magnification (8 mm apochromat N.A. 0.65), ×500.

wanted, a lens with a higher N.A. must be used (see Figure 5).

TABLE 2

Usable Magnification for Some Common Objectives

Common Objectives	Initial Magnification	Numerical Aperture	Working Distance	Usable Magnification
Achromat (40 mm)	3×	0.12	35 mm	×120
Achromat (16 mm)	10×	0.25	16 mm	×250
Apochromat (8 mm)	20×	0.65	0.7 mm	×650
Apochromat (4 mm)	40×	0.95	0.12 mm	×1000
Apochromat (2 mm)	90×	1.30	0.11 mm	×2000

A condition of empty magnification may also result after a photomicrograph has been made. This often happens when an enlargement is made from a negative that is already at its limit of resolution. Any further increase in magnification, such as that produced by an ordinary photographic enlarger, will result in empty magnification. If carried to the extreme, say 5× to 10× enlargement, the print image will exhibit very poor quality.

When a photomicrograph is to be reproduced as an illustration in a book, magazine, etc, the best practice is to utilize a higher-than-necessary, but efficient, magnification in making the photographic negative or original. From this original an illustration can be prepared about 1/5 larger than the final journal reproduction size. This practice seems to minimize loss of image quality when the illustration is reduced to 4/5 its size in the reproduction process.

DEPTH OF FIELD

In ordinary photography, depth of field is considered as the distance from the nearest part to the farthest part of the subject in acceptable focus. When the subject is a considerable distance from the lens, depth of field is measured in feet, but as the subject is brought closer to the lens, the depth of field decreases rapidly. The image of the subject, however, is always smaller than the subject itself.

In photomicrography, as in microscopy, the image is considerably greater in size than the specimen. Depth of field is exceedingly short and is expressed in micrometers. The higher the magnification and the higher the numerical aperture, the shorter the depth of field. The following tabulation shows the approximate depth-of-field figures for dry objectives of various numerical apertures. Depth of field also decreases as wavelength decreases; so computation for only one wavelength, green, is shown here.

Variation in Depth of Field with Change in N.A.

N.A.	0.25	0.30	0.50	0.65	0.85	0.95
Depth (in μm)	8.00	5.50	2.00	1.00	0.25	0.10

It is clear from the table that depth of field is extremely small for objectives of high N.A. Focus of the image with objectives of high N.A. becomes very critical, and just a touch of the fine-focus adjustment on the microscope may cause the image to go out of focus. This very limited field-depth also presents a problem when the specimen is too thick for the objective in use. The out-of-focus areas within the specimen will scatter and diffuse the light passing through, affecting the recorded image quality in photomicrography. Ideally, the specimen should be no thicker than the usable depth of field for a given objective.

The problem is not so acute when the specimen is examined visually in the microscope, since the eye can rapidly accommodate for the actual depth. Then, too, the microscopist constantly changes focus from top to bottom of the specimen in order to see the entire structure.

In photomicrography, however, the image is recorded on the film in one plane. Any pronounced difference between depth of field and specimen thickness may affect the quality of the recorded image.

Selection of Cover Glass

The quality and thickness of the cover glass are controllable factors of great importance for the best image quality. For best results in photomicrography, cover glasses should be scrupulously clean and within a thickness range of 0.16 mm to 0.19 mm.

This thickness-range recommendation is made because an objective lens is corrected optically for spherical aberration, so it will render the best-quality image when the proper thickness of cover glass is used. This factor is particularly critical with any high, dry objective lens that is corrected for a cover glass 0.17 mm to 0.18 mm thick. An excessive thickness of cover glass produces appreciable spherical overcorrection and loss in image sharpness. Cover glasses that are too thin produce undercorrection for spherical aberration, with similar loss in quality (see Figure 6). A "correction collar" is usually incorporated in the mounting of a high, dry apochromatic objective. It can be adjusted to compensate for cover glasses that range in thickness from 0.15 mm to 0.25 mm. This collar is not included, however, in achromat, fluorite, and most phase-contrast objectives.

Cover-glass thickness is not critical with oil-immersion objectives when the refractive index of the oil is equal to, or very close to, that of the cover glass. This refractive index may vary, however. With immersion objectives of highest aperture (N.A. 1.3 and 1.4), it is still important to use cover glasses within the ideal thickness range. Cover glasses of other-than-specified thickness can be utilized in visual microscopic examinations, but since you cannot always be sure when a photomicrograph will be required, it is good practice to use a cover glass of the correct thickness whenever a microslide is prepared.

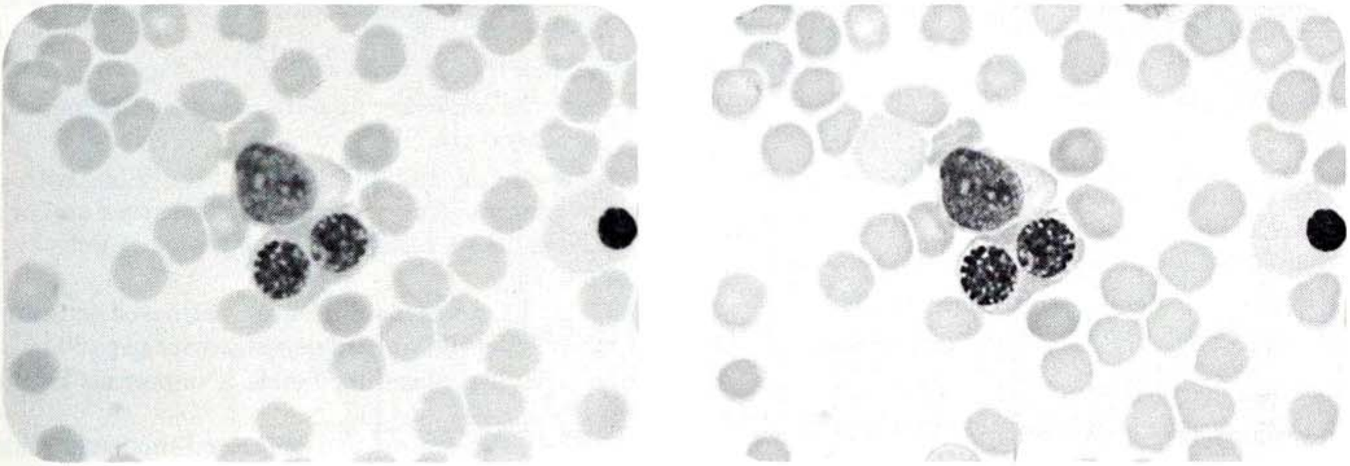


FIGURE 6—(a) Spherical aberration induced by too thin cover glass; (b) Aberration reduced by the use of the proper, specified cover glass.

SPECIAL TECHNIQUES

Microtomy

One important fact that is often overlooked in microscopy and photomicrography is that the specimen is a part of the optical system. The specimen is usually a stained tissue section, a smear, or some other preparation mounted on a microscope slide beneath a cover glass.

It is important that tissue sections be as thin and as uniform in thickness as possible. Although the sections made routinely in most histology laboratories are several μm thick, they would be better for photomicrography if they were made with a thickness in the order of 1 or 2 μm, or less. This, however, is a problem of microtomy and will not be covered in detail here.

Use of Oil-Immersion Objectives

In order to achieve high magnification and high resolving power in a photomicrographic system, it is necessary to use oil-immersion objectives. When an objective is intended for oil immersion, the word "oil" will appear imprinted on the mount. The N.A. of the objective is also shown.

A drop of oil is placed on the cover glass of the specimen slide. If the N.A. of the objective exceeds 1.00, a drop of oil is also used between the bottom of the slide and the top lens of the condenser.

Immersion oil can be obtained from biological supply firms, from microscope manufacturers, or from microscope dealers. Many immersion oils have been found to contain toxic polychlorinated biphenyls. Extreme care should be taken to avoid skin contact or ingestion of these oils. Some nontoxic oils are available.

A bottle of immersion oil should *never* be shaken, since air bubbles may be introduced. A single, microscopic bubble under the objective lens will cause flare, which will lower contrast and affect image quality. Also, the bottle should never be left open to the air, since dust may settle on the oil. Dust or other debris in the oil will impair image quality.

The procedure for using an oil immersion objective is as follows:

1. Search for a suitable field on the slide with a low-power, dry objective. A 10× or 20× objective will suffice.
2. Rack up the coarse adjustment of the microscope and move the oil-immersion lens into position.
3. Place a small drop of oil on the front of the objective and another small drop on the cover glass over the area of interest. This technique will prevent formation of air bubbles.
4. Using the coarse adjustment, move the objective lens down slowly until the oil on the lens contacts the oil on the cover glass. A flash of light will occur at this moment, visible when the eye is close to the level of the lens.
5. Now use the fine adjustment on the microscope to accomplish fine focus of the specimen.

If it is impossible to find the specimen focus, it may be that the cover glass is too thick or that the mounting medium is too thick; in this case, correct focus can never be achieved. Oil-immersion objectives have extremely short working distances.

When the N.A. of the objective exceeds 1.00, oil should be placed on the condenser, as stated. If this is necessary, proceed as follows, *before the objective is focused.*

1. Remove the specimen slide from the stage and place a drop of oil on the top lens of the condenser.
2. Place another drop of oil on the bottom of the slide, under the specimen.
3. Replace the specimen slide on the stage carefully.
4. Rack the condenser up until the oil on the condenser contacts the oil on the slide.
5. Now find a suitable field on the slide with a low-power objective; focus the condenser; and proceed as above, using the oil-immersion objective.

Correct focus of the condenser is achieved according to the Kohler illumination system. (See page 30.) The thickness of the specimen slide is important in this system.

When you are through using the oil-immersion lens, the oil should be removed. Gently wipe the front surface with lens tissue, followed by a tissue dampened with xylol (Eastman Organic Chemical No. T460). Oil can be removed from the slide and the condenser, if necessary, in the same manner.

CAMERAS IN PHOTOMICROGRAPHY

GENERAL CONSIDERATIONS

An efficient microscope, used to best advantage with properly controlled illumination, is the image-forming part of a photomicrographic system. The camera is the means for recording the image formed by the microscope. If the quality of the image is the highest attainable, an excellent photomicrograph can be made. If it is not, then no film, camera, or camera refinement can improve the image quality in the photomicrograph. It is important, however, that the image *should not be degraded* in the camera or by any photographic technique.

Although almost any camera can be used to record a micro-image, a camera specifically designed for photomicrography offers more advantages than one which is merely adapted for the sake of convenience. A discussion of photomicrographic cameras begins on page 17. The selection of a camera is most often governed, however, by the amount of photomicrography to be done and the availability of funds.

CAMERAS WITH INTEGRAL LENSES

The simplest way to make a photomicrograph is to use a conventional, existing camera over the microscope. It might be an inexpensive fixed-focus camera or an expensive 35 mm camera, but it is usually one designed for regular photography of people and places. The lens is an integral part of the camera and often cannot be easily removed. A fixed-focus camera is the simplest, having only one shutter speed and usually only one lens aperture. The more expensive type of camera offers a range of shutter speeds, various distance settings, and a variety of aperture settings. This type, obviously, offers more versatility, particularly in exposure control. Consider this camera first, since it will be more suitable.

When a microscope is focused visually with a normal, relaxed eye, the image is considered to be at infinity. Therefore, the distance setting on the camera should be set at infinity. If the camera is placed over the microscope in the correct position, the micro-image will be in focus on the film plane. Any other distance setting on the camera may cause an unsharp image. Many persons can relax their eyes by staring at a very distant object just prior to focusing the microscope. Spectacles for aiding distant vision or astigmatism should be left on. Should the initial results be unsatisfactory in terms of sharpness, you may be focusing your eye at some other virtual distance such as 15 or 25 feet. When this is the case, a series of photographs should be made which run the gamut of focus settings on the camera. The sharpest record will indicate the virtual distance at which you are most likely to focus the microscope. Those who cannot focus the microscope consistently will have to adopt a camera with a ground glass, preferably with a detachable lens.

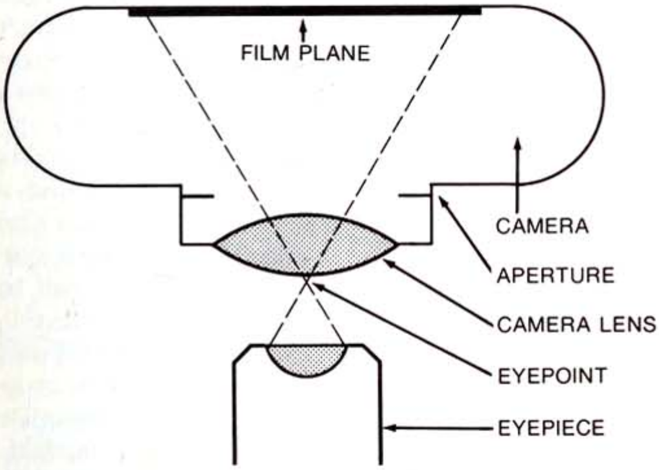


FIGURE 7—The proper positioning of a camera (with integral lens) on the microscope. The eyepoint is focused on the front surface of the camera lens by moving the camera.

The lens-aperture settings (*f*-numbers) on the camera *do not control exposure* in this work as they do in regular photography. They have *no* effect on image brightness. The largest aperture setting should be used—that is, the lens should be "wide open." The effect of using smaller apertures will be to "vignette" the image; that is, to reduce the illumination at the edge of the field because of the restrictive action of the aperture. Also, a stopped-down diaphragm cuts into the image field and reduces field size, so only a small, circular image is recorded on the film.

The camera should be positioned over the microscope so that the "eyepoint" of the eyepiece is at, or very near, the front surface of the camera lens, as shown in Figure 7. The eyepoint is the position *above* the eyepiece at which light rays converge after leaving the eyepiece. They then diverge to form the photographed image. The position of the eyepoint can be determined by holding a piece of white paper right on top of the eyepiece, then slowly raising it. A bright circle will appear on the paper. This circle becomes smaller and then larger. The position at which the circle is the smallest is the eyepoint. The distance of the eyepoint above the eyepiece will vary with different eyepieces. If the eyepiece is changed, therefore, the eyepoint may appear in a different position. The distance may only be a few millimeters or it may be as much as 20 millimeters.

The camera can be held in place over the microscope by any available means. A vertical stand can be constructed out of wood or metal; you can use a laboratory ring stand; or you can attach the camera to the upright member of a small enlarger, with the enlarger head removed. In any case, the camera should be held firmly in the correct position, but you should still be able to move the camera up out of the way or swing it to one side to look into the microscope and adjust image focus. The rule is: Focus the microscope and do not change the distance setting on the camera.

When the image appears sharp, the camera can be replaced in position and the camera shutter actuated to make an exposure. An arrangement must be made to bring the camera back to the correct position. Some kind of "stop" on the stand should be devised.

Now to consider the inexpensive fixed-focus camera. This camera can be placed over the microscope in a similar manner, by using a constructed or improvised stand. There is one important exception, however, with regard to the lens and eyepoint positions. A fixed-focus camera usually has only one, fixed, small aperture behind the camera lens. If the camera is positioned, so that the eyepoint occurs at the front surface of the lens, the small aperture in the camera will reduce field size; only a very small image will be recorded in the camera.

In this case, an eyepiece with a "high" eyepoint is selected. The camera is positioned so that the eyepoint occurs at the aperture. The correct position for a fixed-focus camera is shown in Figure 8. Most fixed-focus cameras will require an eyepiece with an eyepoint distance of about 15 mm.

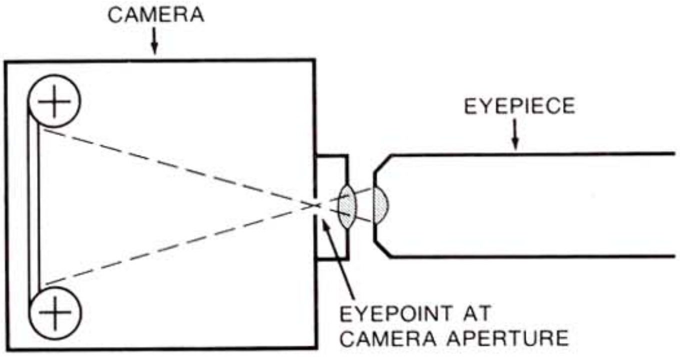


FIGURE 8—Placing the eyepoint at the camera aperture allows the largest field size on film.

Exposure becomes a problem with fixed-focus cameras because usually only one shutter speed is available. With some cameras, two shutter speeds can be used. This means that you must have a very bright image in the microscope and, of course, a very bright light source. The exposure time, or shutter speed, is usually very short and will be about 1/30 or 1/40 second.

A simple method of attaching a fixed-focus or other camera with integral lens is described and illustrated in Kodak Pamphlet No. AN-6, *Photomicrography with Simple Cameras,* available on request from Consumer Markets Division, Eastman Kodak Company, Rochester, New York 14650.

Magnification with a Simple Camera

Normally, when an image is formed outside of the microscope, the magnification, as seen in the microscope (objective power times eyepiece power), is reproduced if the image is 10 inches (250 mm) from the eyepoint. An integral camera lens *always* has a focal length *shorter* than 10 inches. Image (lens-to-film) distance becomes the determining factor for magnification of the photographed image. A camera with a 2-inch (50 mm) lens will record an image only about one-fifth the size of the image seen in the microscope. Magnification on film can then be determined by the ratio between the camera-lens focal length and 10 inches (250 mm), multiplied by the visual magnification of the microscope. Camera lenses, except those on inexpensive cameras, usually have their focal lengths engraved on their mountings. Fixed-focus cameras normally have no designation for either focal length or lens aperture.

To determine the actual magnification recorded on film, you can place a micrometer slide under the microscope (see Figure 4, page 11). The lines of the slide should be focused sharply and the image recorded. When the film is processed, you can measure the separation of the recorded lines and compare the measurement with the actual separation of the lines on the micrometer slide. For example, if two lines on the slide were 0.01 mm apart, and the same recorded lines were 0.5 mm apart, the recorded magnification would be ×50 (0.5 divided by 0.01).

There are other disadvantages to using a camera with an integral lens in photomicrography. One is that the entire microscope field may not fill the film frame. The out-of-focus, peripheral area of the microscope field is then recorded. This condition can be alleviated to some extent by using high-magnification eyepieces so that only the central, best-corrected part of the microscope field will be photographed.

Complex camera lenses will sometimes create internal reflections (due to multiple-element construction). These reflections reduce image contrast in photomicrography. Because simple, inexpensive cameras have fewer lens surfaces to reflect light, they will often produce better photomicrographs than expensive, complex cameras. Of course, a simple camera must be used correctly.

Some camera firms make microscope "adapters" that can be used to place a camera with an integral lens in correct position over a microscope. Such adapters are often made, however, for adjustable-focus cameras, not for the fixed-focus type. Camera manufacturers should be consulted for this accessory to their cameras.

CAMERAS WITHOUT INTEGRAL LENSES

Reflex Cameras

The next type of camera that can be adapted for use over a microscope is the single-lens reflex (SLR). Very often a camera of this type uses 35 mm film, has a focal-plane shutter, and will accommodate a range of interchangeable lenses, from wide angle to telephoto. Many firms that manufacture reflex cameras also offer microscope adapters. Normally, when a reflex camera is to be used over a microscope, the lens is removed from the camera and one or more extension tubes is placed on the camera in the lens position. A microscope adapter ring, containing the microscope eyepiece, is then fastened in the front extension tube. The whole assembly of camera, tubes, and adapter can then be placed on the microscope, fitting the microscope eyepiece and the adapter ring directly into the drawtube of the microscope. This assembly, in some designs, can be attached to a rigid stand that is supported independently of the microscope. This will prevent transfer of vibration from the camera to the microscope.

The micro-image is focused by adjusting the focus knob on the microscope while viewing the image in the camera's viewfinder. One disadvantage of this system is that the image is usually focused on a ground glass within the viewfinder. Critical focus of fine detail is difficult to achieve on a ground glass because of the coarseness of the ground surface. If a clear glass can be used in place of the ground glass, or if a clear area is present on the ground glass near the center, this disadvantage can be overcome.

Bellows Extension Camera

Some firms that manufacture reflex cameras also offer an adjustable bellows, which is normally used in close-up photography. This bellows (with no lens attached) can be used on a reflex camera over a microscope and has the advantage of adjustability, so that magnification and the amount of recorded field can be varied for control of image composition. When the camera is used in this manner, it is advisable to attach the bottom plate on the bellows track to a rigid vertical stand.

With a reflex camera, magnification can be varied, a wide range of shutter speeds is available (usually 1 second to 1/1000 second) for exposure control, and the entire film frame in the camera can be filled. Also, many modern reflex cameras include a "behind-the-lens" metering system for monitoring the image brightness in exposure determination. There are only two drawbacks, or factors, which can affect recorded image quality. One, already mentioned, is the difficulty of critically focusing the image on a ground glass. The other is the possibility of creating vibration when a focal-plane shutter is actuated. This can be minimized by using a shock mounting pad under the microscope. (See also "Camera Vibration" page 20.)

PHOTOMICROGRAPHIC CAMERAS

There are several commercially made cameras designed specifically for photomicrography. The most popular are those called *eyepiece cameras;* a feature generally common to cameras in this group is a beam-splitter eyepiece. The microscopic image can be viewed, focused, and composed by means of this auxiliary eyepiece. A central, rectangular area is often shown in the center of the field to indicate the portion of the field that will be recorded on film (Figure 9).

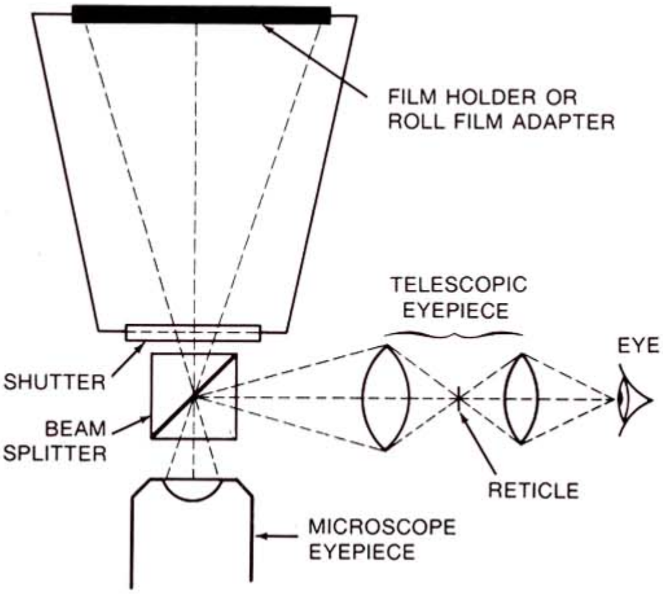


FIGURE 9—Eyepiece Camera—fixed bellows. If bellows draw (the lens-to-film distance) is less than 10 inches (250 mm), a correction lens is often included to adjust the focus to the film plane. The magnification on the film is then the same as the visual magnification.

35 mm Eyepiece Cameras

Cameras of this type usually accommodate 35 mm film magazines, although some use sheet films (or plates). Some with more versatility are designed for use with either roll or sheet film.

The film plane in a 35 mm eyepiece camera is in a fixed position, usually far enough from the microscope eyepiece so that only the central area of the field is recorded. This feature avoids the out-of-focus peripheral area caused by curvature of field, which is inherent in many microscope objectives and which would be recorded in a camera with integral lens. The distance from eyepoint to film may vary slightly for 35 mm eyepiece cameras of different manufacture, but is usually about 4 or 5 inches. Since the distance is less than 10 inches, however, a simple correcting lens is incorporated so that the image seen sharply focused in the viewing eyepiece will also be in sharp focus in the film plane of the camera. The use of this correcting lens also makes sure that the microscope will be used at the correct optical-tube length.

Focusing this eyepiece camera requires care. The reticle (or crosshairs) in the telescopic eyepiece must be in sharp focus when the specimen is in focus. Persons under 40 years old may accommodate a focus difference without realizing it. The best method is to focus the reticle *first*, and then focus the specimen. To check the focus, move your head from side to side while viewing the specimen. If the reticle and a fine structure in the specimen appear to move in opposite directions, a fine focus adjustment is needed. When they seem to move in the same direction and stay together, the focus is correct. This is often called "parallax" focusing.

The greatest advantages presented by an eyepiece camera are convenience and economy, especially when 35 mm slides are desired for projection purposes. This type of camera can be used with most microscopes. It requires little storage space when not in use and can be set up quickly when needed. Any efficient visual microscope setup can be used for photomicrography with an eyepiece camera. This great convenience has made the eyepiece camera very popular and useful for photomicrography.

Roll-film cameras, especially 35 mm cameras, have limitations and disadvantages that should be considered before equipment is purchased for specific use. For example:

1. If 35 mm film is used, a small-size negative or transparency is produced. When prints are required, enlargement becomes necessary, and the extra cost of an enlarger reduces the economy of a small film size. (Also, in enlargement the optical condition of "empty" magnification can occur. See page 11.)
2. Magnification is quite limited on 35 mm film. Some specimens, such as large blood cells, must be photographed at low to medium power because high magnification might make them too large for the film size. This reduces best optical resolution possible, and fine details cannot be well defined because only objectives of lower numerical aperture can be used. Objectives of high numerical aperture are necessary when the best resolution is desired.

Sheet-Film Eyepiece Cameras

Sheet-film cameras of the eyepiece type often reproduce visual microscope magnification, since the eyepiece-to-film distance can be fixed at 10 inches (250 mm). In addition to an observing eyepiece, these cameras often have a ground-glass back to facilitate both composing and focusing of the image to be recorded. Film size is usually 4 by 5 inches. Because of this larger film size, greater magnifications can be recorded with these cameras than with roll-film cameras, especially 35 mm cameras. Also, the same field can be easily exposed on several different emulsions and then developed individually if desired. Adapter backs are available, however, to convert sheet-film cameras to the use of roll films when desired.

Trinocular Attachment Cameras

Another type of photomicrographic camera is shown in Figure 10. This type is especially designed by some manufacturers for use on their "trinocular" microscopes. A trinocular microscope has a binocular arrangement for visual work and a third tube for photomicrography. The visual and the photomicrographic optical systems are "parafocal"; that is, the image seen visually in the microscope will also be in focus in the camera. *A beam splitter is not used.* A 35 mm back and a sheet-film back are interchangeable. Also, a special "field of view" eyepiece is supplied, containing a focusable eye lens and a reticle

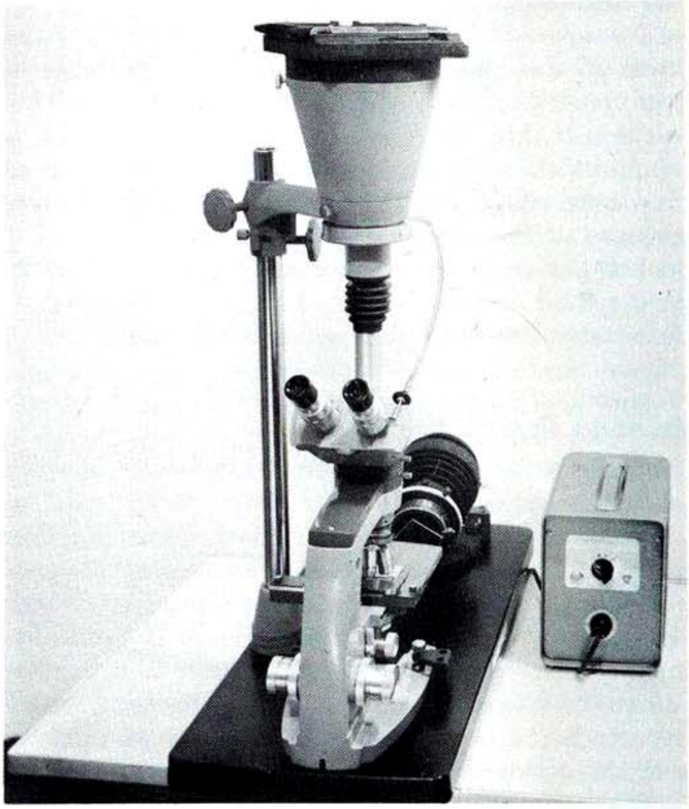


FIGURE 10—Typical trinocular microscope arrangement. A prism deflects the light either to the film plane or to the eyepieces.

to indicate the area of field which will be recorded. Although this is an excellent system for photomicrography, it has one disadvantage—it cannot be used to photograph moving organisms. A prism is used to change the light path from the visual system to the photographic, so it is impossible to see the field while a photograph is being made. If only stationary subjects are to be photographed, there is no problem. The eyepiece camera, on the other hand, can be used to photograph *both* stationary and moving subjects.

Cameras with Adjustable Bellows

A camera that has an adjustable bellows and accepts a large film size is considered best for high-quality photomicrography.

The well-equipped photomicrographer always uses a camera with adjustable bellows. Such a camera usually contains a shutter capable of a wide range of exposure times, a light-locking device to exclude all light from the film except that from the microscope, and a ground-glass screen for focusing and composition. *No* lens is used or needed in this camera. Sheet films or plates in appropriate holders are normally used, but the camera will often accommodate roll films by means of suitable adapter backs. (See Figure 11.)

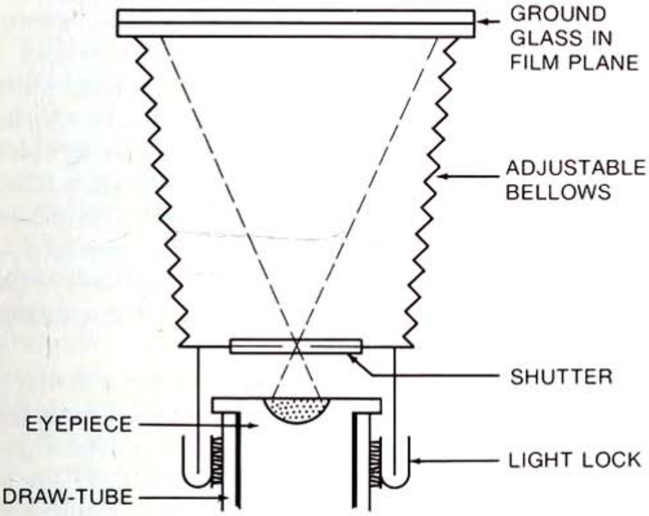


FIGURE 11—Adjustable bellows camera. Focusing the camera is accomplished by adjusting the microscope focus until the image is sharp on the ground glass. The eyepoint is placed at the shutter of the bellows.

Film size is commonly 4 x 5 inches or larger to allow highest magnifications and greatest field sizes. This also allows complete versatility in the selection of objectives and eyepieces.

A camera with adjustable bellows is capable of a complete range of magnification, since eyepiece-to-film distance is adjustable. Microscope magnification is reproduced at the stated 10-inch distance.

Commercially made cameras of this type are usually mounted vertically on a rigid stand (Figure 12).

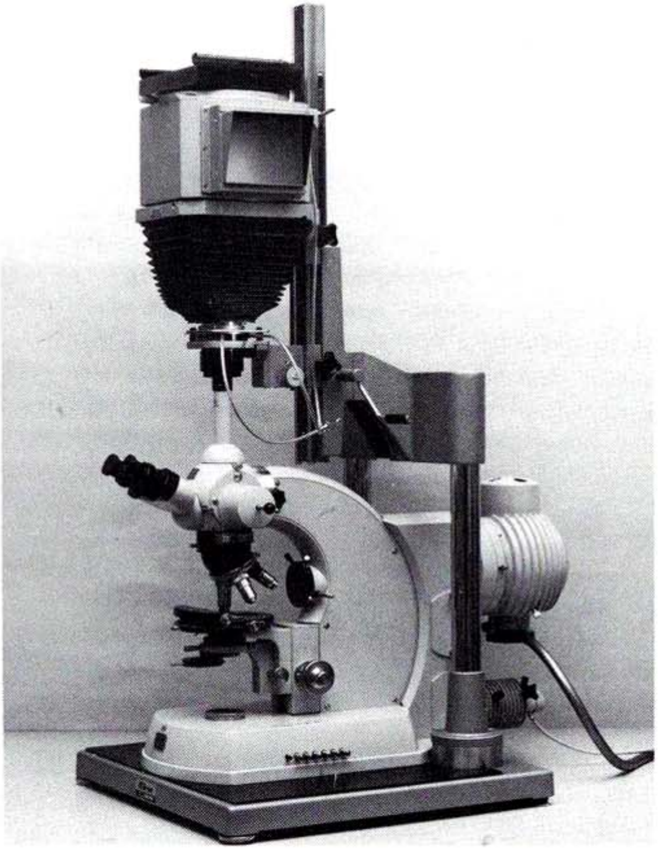


FIGURE 12—Professional photomicrography setup. Light source, optics, and camera are in the same vertical plane.

Actually, a heavy, rigid stand is a necessity in order to avoid vibration and consequent unsharpness in the image. In addition, a separate means of absorbing vibration and shock, such as a vibration isolation table, is recommended for consistently high quality, particularly when long exposure times are indicated.

A precaution that should be observed with this type of camera is correct positioning of the shutter. Ideally, the shutter should have a reasonably large opening and small, light, fast-moving blades. It should be positioned so that the blades are at, or very near, the eyepoint of the microscope eyepiece. If the blades are too far behind the eyepoint and short exposure times are used, a light silhouette-image of the shutter blades may be recorded. (See Plate III-A, page 39.)

Light Lock

Commercial photomicrographic cameras of the adjustable-bellows type usually contain an efficient system for excluding extraneous light from the camera. This is the "light-lock," the connection between microscope and camera. When an available view camera or an enlarger is adapted for this work, a light-lock system must be devised. A very efficient and convenient light lock can be made by using two different-size lens hoods—the smaller one is attached to the camera or shutter and the larger to the tube of the microscope. The two hoods will nest together to exclude all light from the camera except that coming through the microscope.

In practice, the camera is usually moved out of position so that the microscope can be used visually to locate an appropriate field. The camera is then moved into position so that its lens hood fits into the lens hood on the microscope. The camera, of course, should be centered with respect to the microscope so that there is no physical contact between the two lens hoods. Enough space exists to allow movement of the microscope barrel for critical focusing of the image on the ground glass.

Lens hoods in various sizes are standard camera accessories and can be bought in most camera stores.

THE GROUND GLASS

Frequently in photomicrography the image to be recorded consists of extremely fine detail. The surface structure of a ground-glass screen, however, is often so coarse that it interferes with this detail; critical focus of the image is difficult, if not impossible. Some manufacturers of photomicrographic equipment provide screens with clear centers for critical focus of the image on the plane of the ground surface. A ground-glass screen with clear center can also be prepared in the following manner:

With a soft pencil, draw two diagonals on the ground glass surface, which should be facing the microscope. Place a small drop of warm Canada balsam (or other mounting medium) where these diagonals cross. Drop a small, round, micro cover glass onto the balsam and press it into place gently. Allow the balsam to set; a permanent transparent center on the ground glass will result. This procedure provides crosshairs for parallax focusing.

For photomicrography, place a mounted hand magnifier of at least ×5 over the clear center of the screen. Put your eye to the magnifier and glance slowly back and forth over the crossed lines. When the image is critically focused in the plane of the lines, there will be no movement of the image in relation to the crossed lines.

CAMERA VIBRATION

Vibration in a camera setup can result from microscope manipulation, from drawing and replacing dark-slides in film and plateholders, from setting the mechanical shutter and from vibration within the building. It is always good practice to wait a few seconds for vibration to cease before making the actual exposure. Even then, if the shutter is actuated manually, additional vibration can occur and may cause some unsharpness in the recorded image. For this reason, if the shutter is part of the camera, the use of a suitable cable release is strongly recommended. A self-timer can also be used to provide a means of automatic delay before the shutter is operated.

Some cameras contain focal-plane shutters; others have leaf-type, or "between-the-lens," shutters. The latter type is much more satisfactory for photomicrography, since a focal-plane shutter may impart a certain amount of vibration when an exposure is made. This is caused by the shock of the focal-plane shutter striking the side of the camera as the shutter opens and closes. This shock can be alleviated by using longer exposure times to minimize the vibration effect caused by the shutter or by using special absorbing pads or an anti-vibration table under the microscope. These will dampen the vibrations; tests will determine their efficacy. Such units are available from scientific equipment supply firms.

In general, heavy shutters and focal plane shutters on the camera should not be used for timing the exposures. It is better to set them to the "open" position just prior to making the exposure and then to time the exposure with an auxiliary shutter placed in the light beam between the lamp and the condenser.

MICROSLIDES

HOW MICROSLIDES ARE PREPARED

Basically, any specimen to be examined or photographed through a microscope is mounted in a suitable medium on a microscope slide and covered with a cover glass. The specimen is usually colored by means of various biological stains to produce contrast and visualization of structural details. Tissue sections and smears are the most common types of specimens prepared for the microscope, although other subjects can also be suitably prepared.

The actual techniques used in slide preparation can be quite involved and complex, and will not be explained in detail in this book. However, the fundamentals of slide preparation will be reviewed briefly. More detailed explanations can be found in books on microscopy and microtechniques.

A wide selection of prepared microslides is available by mail order from several scientific and biological supply firms. These slides include tissue sections, smears, and many other types of specimens. Prepared slides are used extensively in classroom instruction in biology, botany, medicine, etc.

Slides and Cover Glasses

Normally, a specimen is mounted on a glass slide which is usually 3 inches long and 1 inch wide. All brightfield microscopes are equipped to handle slides of these dimensions. Larger slides are also used, although less often than the 1 x 3-inch size. The larger sizes (1½ x 3-inch and 2 x 3-inch) will accommodate either large sections or a long series of sections.

The surface of the slide should be flat. Also, the glass should be of high quality and chemically stable (noncorrosive), since it may be subjected to many reagents. All commercially available microscope slides are produced with these features and are currently available from a number of supply houses.

The thickness of the microscope slide is of definite importance when high-power photomicrography is considered. Substage condensers are designed for use with slides of specific thickness, within a narrow range of tolerance. Unfortunately, the specifications for slide thickness vary with different microscope manufacturers. One condenser, for example, may require a slide thickness of 1.2 mm, whereas another may specify 1.6 mm. The specification for slide thickness is related to the working distance of the condenser. Slide thickness is less critical for visual microscopy or for photomicrography at lower powers when the top of the condenser is removed. In this case, the condenser has a much longer working distance and the actual slide thickness is unimportant.

If a specimen is to be photographed at high magnification, however, it may be necessary to make a special preparation on a slide of the correct thickness, as specified for the condenser in use. In general, however, a slide thickness of 1.2 mm or less will suffice for most subjects to be examined or photographed at low magnification (up to about 150×).

The cover glass is usually a circle, square, or rectangle of very thin optical glass. Various sizes are available for the different shapes, but if a 1 x 3-inch slide is used, the cover glass should be of suitable size to fit the slide. The most common size for a 1 x 3-inch slide is 22 mm, and can be either round or square. Thus, rectangular cover glasses should be less than 1 inch (25 mm) wide. Smaller sizes are used occasionally for small specimens. Recently, plastic cover glasses have been introduced which should not be used for photomicrography.

Ideally, the thickness of the cover glass should be as specified by all microscope manufacturers, which is either 0.17 mm or 0.18 mm. Cover glasses are available, however, in different thickness ranges as No. 0, No. 1, No. 1½, No. 2, and No. 3. Each number indicates a narrow range of thicknesses, with No. 0 being the thinnest and No. 3 the thickest. The complete range is from less than 0.1 mm to slightly over 0.3 mm. A box of No. 1½ cover glasses includes the ideal range of 0.16 mm to 0.19 mm, with the majority being 0.17 mm. It is suggested, therefore, that whenever a microslide is prepared, the No. 1½ cover glass be used. The only exception would be for preparation of some whole mounts, where a thick cover glass (No. 2 or No. 3) would be of advantage. (For a description of whole mounts, see page 23.) When the right thickness of cover glass is used, the slide can be examined visually and is correct for most photomicrography, particularly with a high, dry objective.

Tissue Sections

A large majority of specimens prepared for the microscope are very thin slices, or "sections," of biological material, usually either animal or plant tissue. Sections are cut on an instrument known as a "microtome," with the rotary type being the most common. A block of tissue is held firmly in a clamp which moves up and down as a hand-actuated wheel is rotated. As the block moves downward, it passes over the edge of a very sharp, stationary knife blade and a thin slice of the material is produced. The thickness of the slice is controlled by a setting on the microtome. Serial sections, one after the other, of the same thickness are cut in this way.

Another type of microtome is used for larger pieces of tissue, and makes large sections. This is the "sliding" microtome, where the block remains stationary and the knife moves back and forth to cut thin slices.

Most tissue specimens, however, are not hard enough to be placed in the microtome and be cut without preliminary preparation. First of all, the tissue is fixed and hardened with a chemical reagent. Then it is dehydrated, cleared, and placed in a bath of molten paraffin wax, where it is left long enough for the wax to impregnate the tissue. The tissue must then be embedded in a block of paraffin. This is accomplished by placing the impregnated tissue in a box-like mold containing melted paraffin. The mold is cooled rapidly by immersing it in cold water to harden the paraffin. The result is a block of wax containing the tissue specimen. This block is trimmed to a convenient size and attached to a holder, which is inserted in the microtome clamp.

The actual techniques of specimen preparation and sectioning are much more involved than are described here. Complete details are available in pertinent literature on the subject.

Sections are cut on a microtome either individually or as "ribbons." A ribbon is a long chain of sections which cling to each other at the edges. Either one section or a short ribbon is placed on a microscope slide that has been treated with an adhesive material. The paraffin is then removed by bathing the slide in a suitable solvent. The sections fastened to the slide are usually colorless and practically transparent, unless stained (see below) before embedding. Little or no detail would be visible if they were examined in a microscope. In order to render the details visible, the sections must be colored, or "stained"; many different biological stains are used for the purpose. Double staining, that is, the use of two stains, is common in order to differentiate between specific parts of a specimen. For example, with animal tissue the nucleus of a cell might be stained with one color and a stain of different color used for the cytoplasm, the area of the cell around the nucleus. The most common stains used for animal tissue sections are "hematoxylin" and "eosin." (See Table 5, page 33.) These stains appear as blue and light red, respectively.

Many other colored stains are also used in both plant and animal histology. Some techniques utilize triple, quadruple, and even quintuple staining. Single stains are used rarely.

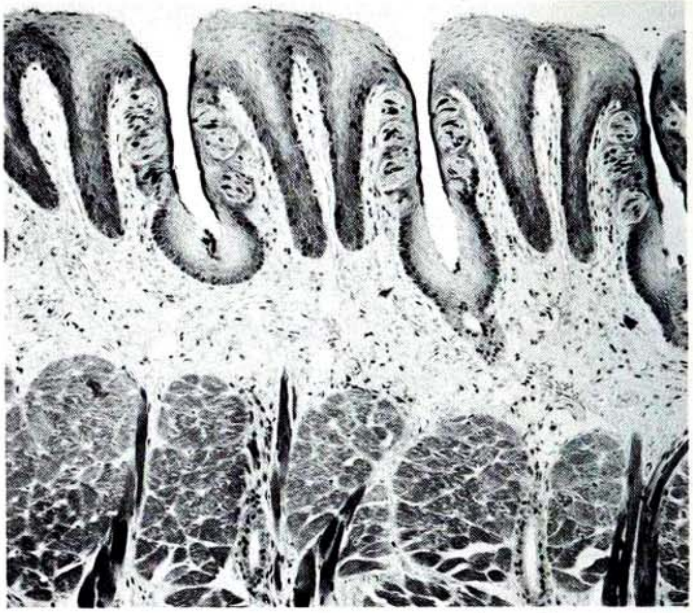


FIGURE 13—Same as Plate I (page 37) on KODAK EKTAPAN Film. A yellow filter (KODAK WRATTEN Filter, No. 12) was used to lighten stain color and to emphasize taste buds.

When tissue sections are to be prepared, it would be best for photomicrography if they were as thin and as well stained as possible. As discussed previously, thick sections present a problem when photomicrographs are to be made at medium to high magnification. There are understandable limits, however, in the preparation of thin specimens by the paraffin technique. Sections thinner than 4 or 5 μm are difficult to make. However, sections as thin as 1 or 2 μm are needed for high-magnification work, both visually and photographically.

In order to make these ultra-thin tissue sections, it is necessary to resort to a different impregnating and embedding substance than paraffin, as well as to use a different technique for preliminary preparation of the tissue. The media most commonly employed in place of paraffin are the methacrylate and epoxy resins. Sections are cut on an "ultra-microtome" by either a glass or a diamond cutting edge. This technique is commonly used in preparing sections for electron microscopes.

Another technique for sectioning tissue for the light microscope involves quick-freezing. Tissues may be hardened sufficiently by freezing so that the usual infiltration and embedding techniques are eliminated. The material to be sectioned is fresh and can be either fixed or left unfixed. The principal advantage, of course, is speed, since the steps of freezing and sectioning can be carried out in minutes—compared with hours for the paraffin technique. The disadvantages are relatively thick sections, less clarity of detail, and the inability to handle large pieces of tissue because they do not freeze entirely throughout. In pathology, however, the ability to make stained, mounted specimens of human tissue in a very short time is a definite advantage.

No matter how a section is made it is mounted on a microscope slide, stained; a drop of suitable mounting medium is placed on it; and then the preparation is covered with a cover glass. The microslide is then ready for viewing or for photography through the microscope.

Smears

The technique of preparing certain types of specimens by "smearing" is relatively simple compared with making tissue sections. A sample of fluid material—such as blood, a bacterial culture, or an exudate—is spread to a thin layer on a clean microscope slide. The smear is then dried and fixed (made permanent). A suitable mounting medium is applied and the specimen is covered with a cover glass.

The methods of smearing will vary slightly for different types of specimens. Blood smears are the most common and are made routinely in hematology laboratories. Two methods of making blood smears are practiced. In the first method, a drop of blood is placed on a clean slide about 1 inch from the end. A second slide, held at about 45° and in contact with the first slide, is allowed to touch the blood so that capillary action distributes the blood along the trailing edge of the slide. The slide is then pushed forward quickly to produce a thin smear on the bottom slide. The smear is allowed to dry in air and it is ready for fixing.

The second method for blood smears involves placing a drop on a clean cover glass. A second clean cover glass is touched to the drop of blood on the first and then dropped diagonally across the first glass. The drop spreads quickly between the two glasses. The two cover glasses are separated immediately, by grasping two projecting corners and pulling with a smooth motion. Each cover glass then contains a smear which can be dried in air, then fixed and mounted on a clean slide.

Wright's Stain, a combination of methylene blue and eosin, is dissolved in methyl alcohol and commonly used for fixing smears in the United States.

Preparation of bacterial smears and smears from exudates is even simpler. A drop of dilute liquid material is picked up with a small wire loop. The drop is then smeared on a clean slide by moving the loop in contact on the slide, either back and forth or in a circular motion. The smear is then dried, stained, and a mounting medium and cover glass applied.

Whole Mounts

There are many specimens which are mounted directly onto microscope slides in specific media without resort to either sectioning or smearing. These include small insects, protozoa, crustacea, pollen grains, and fibers. The techniques of preparation and mounting are quite diverse. The reader is therefore directed to literature on microtechniques, found in most scientific and biological libraries. Many types of whole mounts can also be purchased as prepared slides from supply firms.

The Mounting Media

Micro-specimens for a brightfield microscope are nearly always mounted in some kind of medium, on a slide and under a cover glass. The purpose and characteristics of the medium are variables, depending on the type of specimen. Mounting media basically are divided into two classes: those suitable for permanent mounts and those suitable for temporary mounts.

With regard to stained tissue sections and smears, the mountant is usually permanent; it serves to protect and preserve the specimen for future study or photography. A medium for this purpose must be reasonably colorless, so as to produce a neutral background and not degrade the specimen colors. It should have a refractive index fairly close to that of the specimen in order to produce the highest degree of transparency. It should be chemically inert both to the glass and the specimen, and should not cause stains to fade. Also, it should adhere to glass.

A permanent mounting medium is usually either a natural or a synthetic resin. Canada balsam is probably the most well-known example of a natural resin. It has been used as a mounting medium for over a hundred years, and is still commercially available from many sources. Slides prepared with this medium, however, tend to become slightly yellowish with age, or to appear slightly yellowish when an excess of the medium is used. Some fading of stain colors also occurs after a long period of time.

Synthetic media are employed quite extensively today, and are available under a variety of trade names. They are considered in some ways to be superior to balsam and are often used as substitutes. Table 3 lists some of the common mounting media.

Occasionally, a permanent medium is selected which has a higher refractive index than either the glass or the specimen. In this case, the aim is to make the specimen more visible, since it may be colorless (unstained) and practically transparent. The greater the difference in refractive index between the medium and the specimen, the higher will be the visual contrast. One application is in fiber microscopy, especially with animal hairs. The surface texture of wool fibers, for example, is much more visible in a medium of high refractive index than in one where the index is close to that of the specimen. Some media of this type are Aroclor resins, Naphrax, and polystyrene. (See Table 3.)

When permanent mounts are prepared, a quantity of medium is placed on the specimen and covered with a cover glass. Sufficient pressure must be applied, either by a weight or by a spring-loaded press, to push the cover into place. The cover-glass surface must be parallel to the glass slide, and a minimum amount of mounting medium should exist between the cover and the specimen. The slide must be kept under pressure until the mounting medium has hardened. The slide is then ready to examine and photograph.

Temporary mounting media are often used with specific subjects for speed and convenience. A slide made with a temporary mount is often discarded after use. Some of the temporary fluid media include water, glycerine, certain oils, Karo syrup, and many organic liquids. One precaution which must be observed is that the specimen should not be soluble in the medium or be reacted upon by it. The cover glass is usually taped down to prevent movement.

Chemical Crystals

The formation and photomicrography of chemical crystals is fascinating, particularly when you use either a polarizing microscope or a conventional brightfield microscope equipped with polarizing filters. Some crystals are "birefringent," or "anisotropic," and appear brightly colored when viewed between crossed polarizers. Very striking color photomicrographs can be made of crystals or crystal patterns by using polarized light.

The preparation of chemical crystals on a microscope slide is relatively easy. The simplest method is by evaporation. Dissolve a small amount of chemical in distilled water (or other solvent) in a test tube or small vial. Place a drop of the solution on a clean microscope slide and allow the solvent to evaporate. Crystals will begin to form in a short time. (Application of low heat will hasten crystallization.) The growth can then be studied under a low-power microscope with crossed polarizers. When all of the solvent has evaporated, crystals can be photographed dry, or a mounting medium of *high* refractive index can be applied and a cover glass used. The mounting medium can be a permanent type, but its solvent should not attack the crystals. This might happen with some organic chemicals but is unlikely with inorganic materials. (See Table 3.)

Fusion is another technique which produces color-

TABLE 3

Common Mounting Media

Mounting Media	Refractive Index	Use	Available From
Canada Balsam	1.53	Biological, general	Scientific supply firms
Caedax	1.58	General	Ward's Natural Science Establishment, Inc.
Diaphane Green	1.54	Especially recommended for hematoxylin-stained specimens	VWR Scientific, Inc.
Harleco Synthetic Resin	1.52	General	Hartman Leddon Company
Permount	1.54	Biological, general	Fisher Scientific Co.
Aroclor Resins	1.63	High refractive index work	Monsanto Chemical Co.
Carmount 165	1.65	High refractive index work	R.P. Cargille Laboratories, Inc.
Castor Oil	1.47	Temporary mounts, general	VWR Scientific, Inc.
Cedar Oil	1.52	Temporary mounts, general	VWR Scientific, Inc.
Immersion Oil	1.51	Temporary mounts, general	R. P. Cargille Lab.
White *Karo* Syrup	1.42	Semipermanent mounts, ultraviolet	Grocery stores

ful crystal patterns under polarized light. A very small amount of an organic chemical is placed on a microscope slide and a cover glass placed on top. Then, if heat is applied to the bottom of the slide, the chemical melts and spreads evenly under the cover glass. The heat is removed, and as the melt cools, crystal growth begins when the temperature drops below the melting point. All of the crystals formed will "fuse" together, very often producing beautifully colored patterns.

Only a low-power microscope is necessary for this photography; either a 5× or 10× objective will usually suffice. Crystal patterns are often too thick for higher power.

HOW MICROSLIDES ARE ILLUMINATED

A complete understanding of the principles and practices of obtaining efficient illumination is just as important to the photomicrographer as knowing the capabilities and limitations of the compound microscope. Correct adjustment of the optical system of the microscope is, in fact, dependent upon an efficient system of illumination. An objective, for example, cannot be used effectively unless the substage condenser is properly adjusted and the substage diaphragm is set at the correct aperture. These adjustments are made by following the system known as Kohler illumination. (See page 29.)

The light source itself should provide sufficient intensity to allow reasonably short exposure times. The lamp housing should be suitably designed to allow easy access to the light source and should contain those elements necessary for proper adjustment of the illumination furnished to the microscope.

When a color film is to be exposed, the light source should conform to, or allow suitable filtration to meet, the requirements of the film. A source with a continuous visible spectrum is necessary. Most common light sources meet this requirement. The only exception is the mercury-vapor lamp, which emits line spectra. This source, however, is of special interest for ultraviolet and fluorescence photomicrography. It can also be used in regular black-and-white work.

Efficient illumination is not only dependent upon correct, suitable adjustments of the microscope and the illuminator, but is very much dependent on correct alignment of all components of the system—from the light source to the film plane. Many effects of uneven illumination in the image, especially color fringes, can be traced to improper alignment. If centering devices are not provided for the condenser and the light source, alignment already has been established by the manufacturer. The microscope manual or manufacturer should be consulted if alignment problems persist.

Light Sources

The most common light source in general brightfield photomicrography is the incandescent *tungsten filament lamp,* available in a wide selection of voltages and wattages. Most microscopes having built-in illumination utilize either a 6- or 12-volt coil filament lamp, which varies in color temperature from about 2700 K to 3200 K, depending on manufacture and electrical conditions at the time of usage. Operation of this low-voltage lamp is through a transformer having several settings. The highest setting is usually suggested when color film is to be exposed in order to provide the highest color temperature. Even when a color film balanced for tungsten illumination is to be exposed, it is usually necessary to use appropriate light-balancing filters to adjust the illumination so that it will be right for the film. These filters will vary with different lamps.

When a microscope does not have built-in illumination, an external illuminator must be used. Separate illuminators are available from microscope manufacturers or dealers. These illuminators contain either a 110-volt, 100-watt, coil-filament lamp or a 6-volt, 1.8 amp. ribbon-filament lamp. Here again, however, the illumination must be adjusted with appropriate filters to suit the color film in use. (Plate I-B, page 37.)

One of the problems involved with a tungsten lamp is that the glass envelope becomes blackened with age, due to the deposit of tungsten resulting from vaporization. This effect causes a reduction in light intensity and a drop in color temperature. When a lamp has become visibly blackened, it should be discarded and replaced with a new lamp.

This problem does not exist with the *tungsten-halogen lamp.* Although it is also a tungsten coil-filament lamp, halogen gas is contained within the envelope. When the lamp is ignited, tungsten evaporates from the filament and is intercepted by the halogen gas particles. These combine because of the heat involved, and the tungsten is redeposited upon the filament. The halogen gas is released and the cycle begins again. Deposition of tungsten (on the glass envelope) is thereby eliminated, and the lamp retains its initial brightness and color temperature (3200 K) throughout its life. Tungsten-halogen lamps are available in suitable housings from some microscope manufacturers. New microscopes having built-in illumination from a tungsten-halogen lamp have been developed.

The tungsten-halogen lamp—available at present in a 12-volt, 100-watt size—is an excellent source for photomicrography. It emits high-intensity, efficient illumination because the coil filament is small and compact. Lamp life is about fifty hours, and it can be replaced easily and inexpensively.

The *xenon arc* is another light source that can be used in photomicrography. This lamp produces illumination of high intensity and of daylight quality. The latter feature is especially important, because it allows the use of daylight-type color films with little or no filtering. The arc is produced across tungsten electrodes in a clear envelope which contains highly pressurized xenon gas. The emission of the xenon arc is continuous not only in the visible spectrum but also in the long-wave ultraviolet and infrared spectral regions. Xenon-arc lamps operate from a special power supply connected to a 110- to 120-volt source.

The *zirconium arc* is another excellent light source. It is very small, being almost a point source. Its color temperature is 3200 K, and therefore it is quite suitable for color films balanced for 3200 K illumination. The light intensity of a zirconium arc is not as high as tungsten-halogen or the xenon-arc lamps, but is high enough for efficient photomicrography. The 100-watt size is usually suggested, although lower wattages are also available. Like the xenon arc, it operates from its own power supply.

Electronic flash lamps can also be used in photomicrography, specifically in photographing moving organisms. The illumination is of daylight quality and therefore is satisfactory for use with daylight-type color films. Since the flash is instantaneous, an auxiliary tungsten lamp of low brightness is usually necessary for purposes of alignment, for producing Kohler illumination, for focusing and composing the specimen image, or for general viewing of the specimen prior to photography. The flash should be synchronized with the operation of the camera shutter.

Mercury-vapor lamps serve as excellent monochromatic-light sources. With appropriate filters, the mercury green line at 546 nm, the blue line at 436 nm, or the 365 nm line in the ultraviolet can be used for monochromatic black-and-white photomicrography. A mercury-vapor lamp should not be used in color photomicrography with a brightfield microscope, since its illumination only contains line spectra, and thus, will not give a true rendition of the subject.

Illuminators

Illuminators for visual microscopy and for photomicrography can be obtained from all major optical companies, particularly from those that manufacture microscopes. Some requirements for a photomicrographic illuminator are not absolutely necessary when only visual microscopy is intended. Generally, however, a brighter light source is required for photomicrography than for visual work.

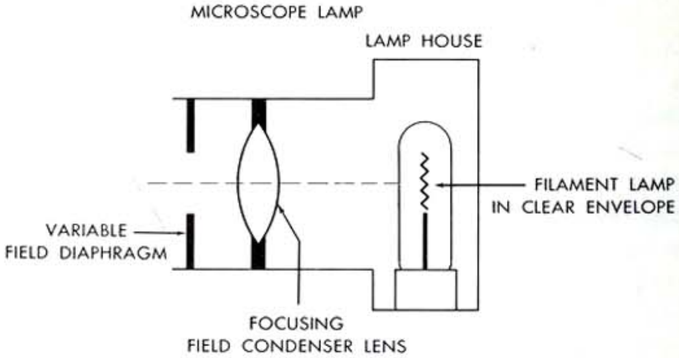

MICROSCOPE LAMP
LAMP HOUSE
FILAMENT LAMP IN CLEAR ENVELOPE
VARIABLE FIELD DIAPHRAGM
FOCUSING FIELD CONDENSER LENS

FIGURE 14—Typical lamp used in photomicrography.

Because complete control of illumination is necessary, photomicrographic illuminators should contain both a lens to project an image of the lamp filament and a diaphragm to control the size of the illuminated field in the microscope. The lens is usually called a "field condenser"; the diaphragm, a "field diaphragm." Also, the terms "lamp condenser" and "lamp diaphragm" are used. (See Figure 14.)

Another refinement, not always included on an illuminator, is a facility for centering the light source with respect to the field condenser lens. We often assume that a light source in a lamphouse *is* centered, but this is not always true. Lamps, themselves, vary in regard to the position of the filament in the envelope. A centering facility corrects any discrepancy.

Since filters are almost always used in photomicrography, a filter holder should also be included on the illuminator, in front of the field condenser lens.

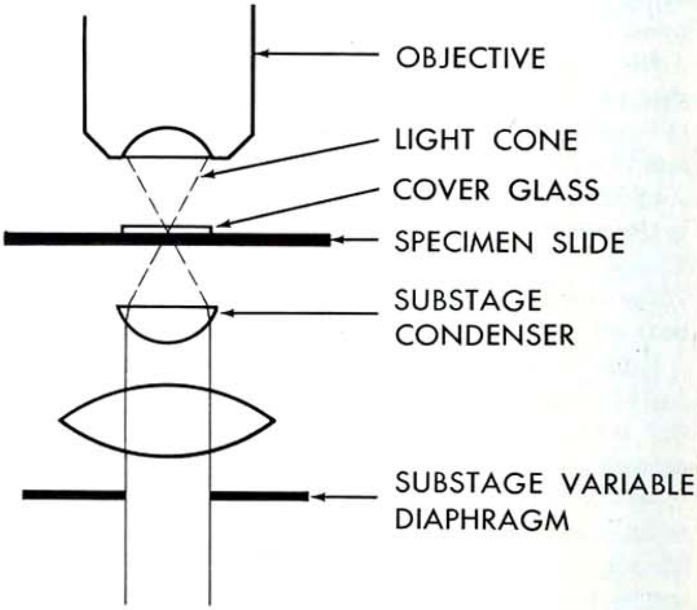


FIGURE 15—Cone of light from substage condenser with apex in specimen plane. Inverted cone fills aperture of objective. Critical focus of condenser is necessary.

Methods of Illumination

There are two ways to illuminate specimens—by transillumination and by reflected light. The second is used for photographing the surfaces of thick or opaque subjects. It is accomplished with microscope lamps that project a small spot of light or by special built-in ring or axial systems. Metallography utilizes the latter and this field is discussed later in this book. The majority of subjects encountered in general photomicrographic work are examined and photographed by transmitted illumination.

The simplest setup involves the use of axial transmitted light. Rays of light are reflected from the plane surface of the substage mirror through the substage condenser, through the subject and the field around it, and into the microscope objective. (See Figure 15.)

A movable substage condenser permits accurate control and concentration of the light and the use of high-power objectives. The illumination should fill completely the diaphragm opening of the condenser with light of even intensity. The condenser must be properly adjusted to insure that the back lens of the objective is filled evenly with light. (See Figure 16.) The plane, or flat, side of the substage mirror should always be used when a condenser is employed. The spherical mirror is used only when a 48 mm objective is used without a condenser.

If the illumination system has not been properly adjusted, a photomicrograph may be disappointing—even if the highest quality optics have been used, the image has been focused critically, and the exposure of the film has been correctly determined. The two basic requirements are (1) that the whole illumination system be centered, and (2) that the cone of light from the illumination system completely fills the aperture of the microscope objective, providing uniform illumination over the whole subject field.

Kohler illumination is the most common system of illuminating a microscope specimen in photomicrography. It can be used in visual work, too, because it provides best image quality and highest resolution.

Basically, Kohler illumination consists of using the field or lamp condenser to focus an image of the lamp filament on the substage condenser, which in turn focuses an image of the lamp condenser in the plane of the specimen. Thus, in effect, the lamp collector becomes the source of illumination. (See Figure 17.)

The chief practical advantage of this technique is that, when the elements are properly aligned, a uniformly illuminated field is provided, with practically no restriction as to the structure of the light source. Hence, it is possible to employ a nonuniform source (such as a coil-filament tungsten lamp) or a high-intensity source of small area (such as a zirconium arc

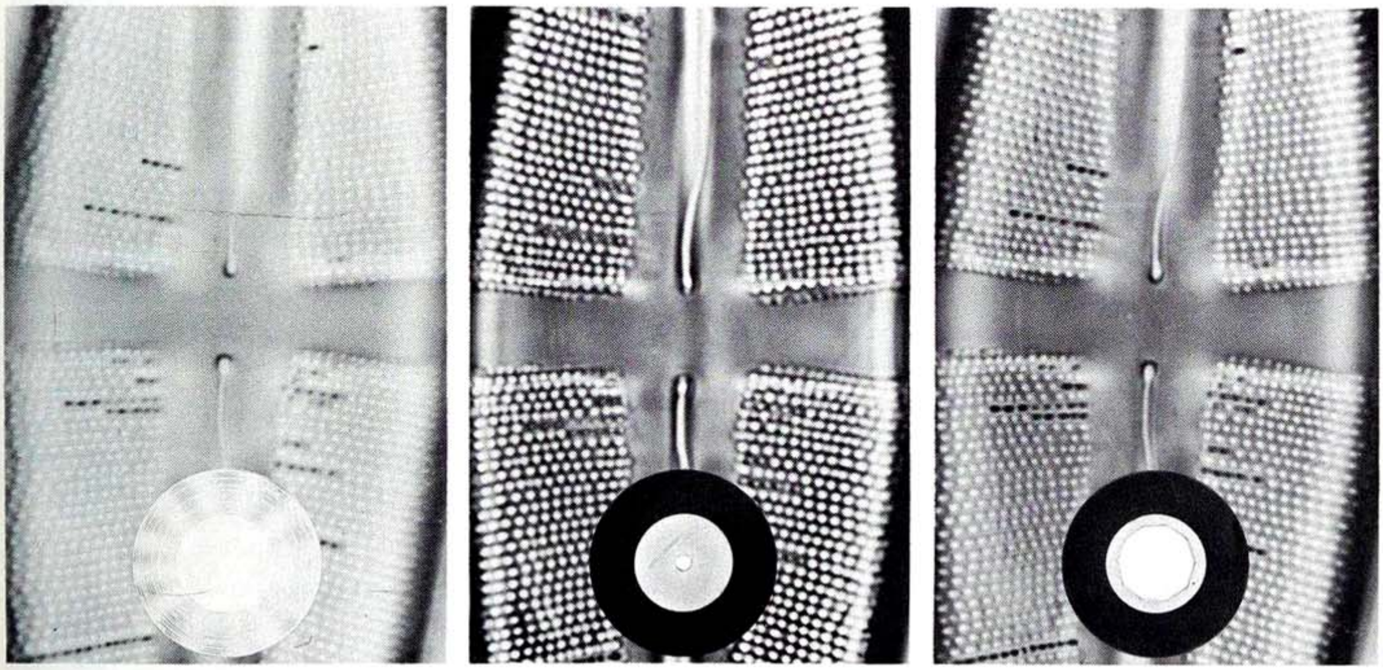


FIGURE 16—Adjusting the aperture diaphragm for optimum resolving power of the objective (a) wide open, (b) stopped down, (c) correct aperture. The bright area should be 4/5 the diameter of the distant circle.

or a xenon arc).

Essentially, the method of producing Kohler illumination in photomicrography consists of focusing an image of the lamp collector in the plane of the object and an image of the lamp filament in the plane of the aperture diaphragm. Refer to Figure 17. In actual practice, the steps in establishing Kohler illumination are listed below and in the following section:

1. Check alignment of the lamp with respect to the lamp collector. A quick check of alignment can be made by placing a piece of thin white paper over the field diaphragm and focusing the lamp collector to produce an image of the lamp on the paper. The image of the lamp should be centered within the outline of the diaphragm. The lamphouse—containing the lamp, collector lens, and diaphragm—should be placed about 10 inches from the microscope. The actual distance should be such that the image of the lamp filament projected onto the mirror is slightly larger than the maximum opening of the aperture diaphragm. The alignment of the lamp with respect to the lamp collector is necessary only once. When the lamp is changed, realignment is required.
2. Adjust the lamphouse so that the image of the lamp is centered on the mirror. Adjust the mirror so that the image reflected by it onto the aperture diaphragm is centered on the diaphragm. Focus the lamp collector to produce a sharp image of the lamp filament on the aperture diaphragm.
3. Stop down the field diaphragm to a small aperture. While looking into the microscope or at the ground glass of the camera, focus the condenser to obtain a sharp image of the field diaphragm in the specimen plane. For this step, the microscope must be in focus on a specimen. (See "Chromatic Aberration," page 43.)
4. The image of the field diaphragm should be centered in the observed field. If it is not centered, alignment of the lamp to the mirror or lamphouse adjustments were not properly made and should be corrected.
5. While still observing the diaphragm image, open the field diaphragm until its diameter is equal to, or just slightly greater than, the entire microscope field as seen.

Having accomplished the requirements of Kohler illumination, it is necessary to take full advantage of this illumination. The back aperture of the objective lens must be filled to obtain maximum resolving power by adjusting the aperture diaphragm.

Adjusting the Aperture Diaphragm

The aperture diaphragm is located beneath the condenser and is adjustable. Its purpose is to control the diameter of the light beam entering the condenser, thereby controlling the angle of the cone of light entering the objective lens so that the full aperture is utilized. The aperture diaphragm is adjusted in the following manner (step numbers are continued from previous section):

6. Focus on the specimen and remove the eyepiece from the drawtube of the microscope.
7. Look down the tube. The back lens of the objective will be visible as a bright circle. (This step is aided by the use of a pinhole eyepiece.) The centered lamp-filament image (Step 4, above) will also be visible.
8. Reach below the condenser and adjust the opening of the aperture diaphragm until its edge is seen within the bright circle. The correct setting occurs when the diameter of the diaphragm image is about 0.8 that of the back lens image, as seen in Figure 16.

If the edge of the diaphragm image is not visible, the aperture diaphragm is open too far. If it is left this way, the effect will be a loss of contrast in the image of the specimen. Poor image quality will result.

If only a very small diaphragm image is visible, the diaphragm opening is reduced too far. The effective numerical apertures of the objective and condenser are thus reduced, and poor resolution results. Also, pronounced interference fringes appear in the specimen image. Such fringes produce low image quality.

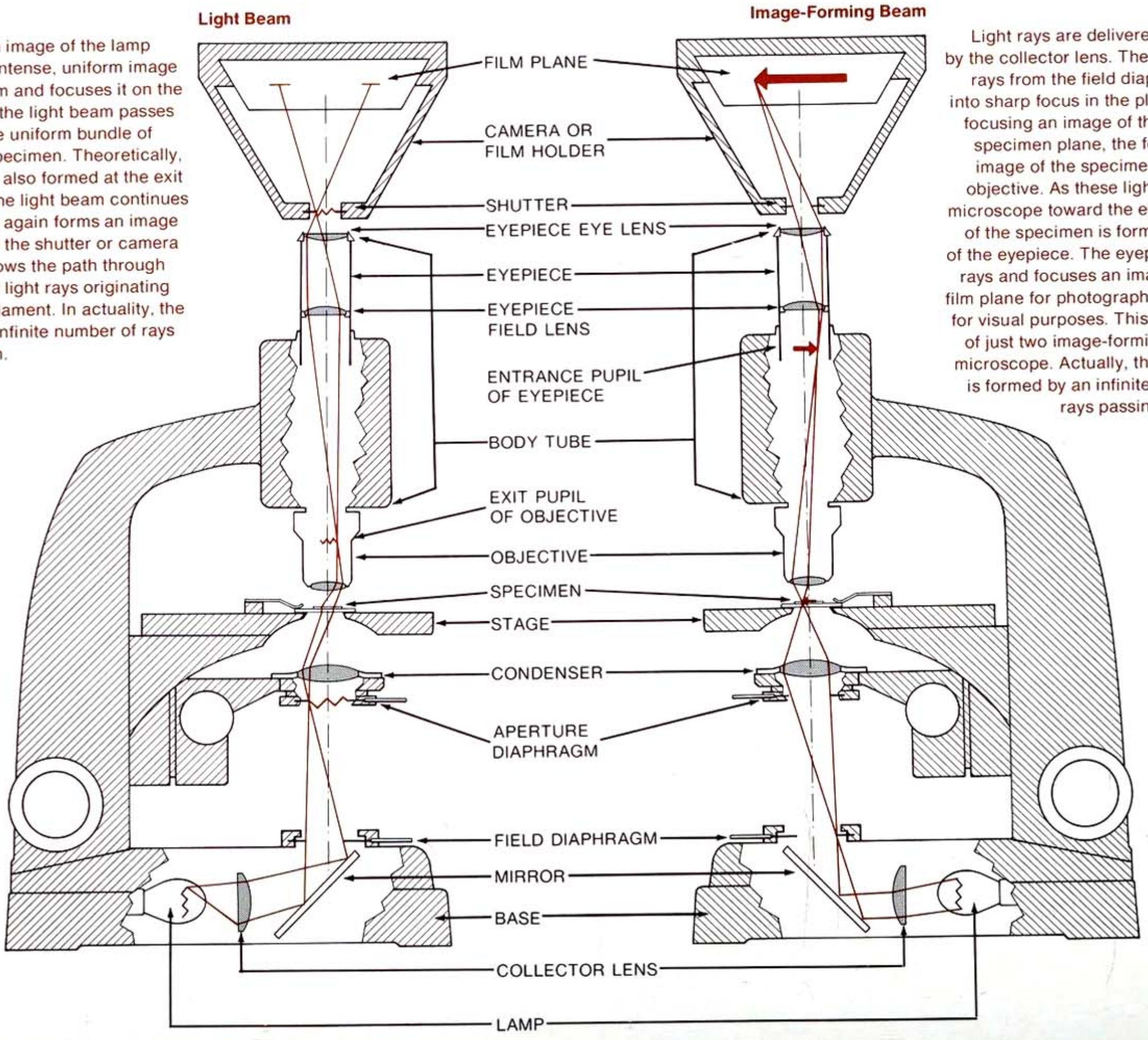
The effect on image quality for three settings of the aperture diaphragm are shown in Figure 16. Proper adjustment of this diaphragm cannot be overemphasized if the best image quality is desired.

Some microscopes have numbered settings marked on the edge of the aperture diaphragm. Since each objective will require a different setting, this facility enables one to adjust the diaphragm accurately without looking down the tube of the microscope. Each time the objective is changed, the correct, predetermined setting can be made easily. It is also possible to mark the diaphragm with the correct setting for each objective.

Adjusting the Field Diaphragm

The principal functions of the field diaphragm are control of image contrast by minimizing flare and control of the size of the illuminated field. This diaphragm has no effect on image brightness. If brightness is affected, the microscope is not properly adjusted for Kohler illumination.

A collector lens gathers an image of the lamp filament and projects this intense, uniform image through the field diaphragm and focuses it on the aperture diaphragm. After the light beam passes through the condenser, the uniform bundle of light passes through the specimen. Theoretically, an image of the filament is also formed at the exit pupil of the objective. As the light beam continues through the microscope, it again forms an image of the filament, this time at the shutter or camera aperture. This diagram shows the path through the microscope of just two light rays originating at the same point on the filament. In actuality, the collector lens focuses an infinite number of rays on the aperture diaphragm.



Light rays are delivered to the field diaphragm by the collector lens. The condenser receives the rays from the field diaphragm and brings them into sharp focus in the plane of the specimen. By focusing an image of the field diaphragm in the specimen plane, the focused light picks up an image of the specimen and projects it into the objective. As these light rays travel through the microscope toward the eyepiece, an aerial image of the specimen is formed at the entrance pupil of the eyepiece. The eyepiece receives the image rays and focuses an image of the subject on the film plane for photography or on a viewing screen for visual purposes. This diagram shows the path of just two image-forming light rays through the microscope. Actually, the image of the specimen is formed by an infinite number of focused light rays passing through the specimen.

FIGURE 17—Kohler illumination is a method of brightfield illumination necessary to successful photomicrography. Establishing Kohler illumination provides a uniformly illuminated field from a nonuniform source such as a filament lamp. The paths of the light beam and image-forming beam are diagrammed here.

In photomicrography, the field diaphragm is adjusted until the diameter of its image is equal to, or just larger than, the diagonal of the film size. Its image can be seen and adjusted in size while looking either through a beam splitter or at the film plane of the camera (as on a ground glass).

If the micrsocope is only used for visual work, the field diaphragm can be opened until the entire circular field is seen in the microscope.

Condenser

As stated previously, an achromatic condenser is highly recommended for all brightfield photomicrography, but particularly for use with color films. It is essential, however, that the condenser, whatever the type, be accurately centered with respect to the objective for good photomicrography. Loss of light and uneven illumination can result when the condenser is out of alignment.

Since specimen slides may vary in thickness, it is usually necessary to readjust the position of the condenser correctly each time the slide is changed.

The best slide thickness is usually specified by the manufacturer for each condenser. Slide thickness becomes quite critical when the use of immersion oil is required between the bottom of the slide and the top lens of the condenser. If the slide is too thin, oil contact is lost when an attempt is made to focus the condenser according to the Kohler system. If the slide is too thick, it may be impossible to focus the condenser correctly.

Built-In Illumination

The present tendency in the production of modern microscopes is toward more convenient and compact illumination systems. Many microscopes (American, European, and Japanese) include illuminators built into the base. The illumination provided to the specimen is usually a modified type of Kohler illumination, in that either a diffusing disk or a lens with a diffuse surface is placed in front of the light source. No adjustment is provided for either the lamp position or the field condenser. As a result, the lamp-filament image cannot be projected to the plane of the aperture diaphragm. Centering screws, however, are usually included for the lamp itself so that its position can be adjusted for even illumination.

The entire illumination system is fixed in place, and the lamp and the collector lens are so positioned that an image of the diffuser is formed on the diaphragm. A field diaphragm is provided so that its image can be formed in the specimen plane by adjusting the condenser. An aperture diaphragm is also included; its setting should still be established correctly, as discussed on page 28.

Low-wattage tungsten lamps are generally used in built-in systems. The illumination provided to the specimen must always be corrected with appropriate light-balancing filters if color films are to be exposed. It should be noted that built-in or separate systems intended only for visual observation are not suitable for photomicrography. An example is a form of opal glass illuminator used for making blood-cell counts.

Image Brightness and Neutral Filters

Very often when the microscope and the illumination are correctly adjusted, the image in the microscope will be extremely bright—too bright, in fact, for comfortable visual observation. The brightness of the image can be reduced by placing a neutral density filter in the light beam. The degree of brightness will indicate how dense the neutral filter should be in order to provide a comfortable level of brightness. Usually, a density of 1.00 or 1.20 will suffice; many built-in illumination systems incorporate such a neutral filter for this purpose. Generally, it should be removed for photography, since it will prolong exposure time.

When a separate microscope illuminator is used, it is good practice to have a neutral filter available to reduce image brightness for visual work. In this case, the neutral filter is placed in a filter holder on the front of the illuminator, and can easily be removed when a photomicrograph is to be made.

Although closing the aperture diaphragm decreases image brightness, image quality suffers for both visual and photographic purposes.

Neutral filters of several densities are actually used in photomicrography to control exposure time so that it will be within the range of available shutter speeds.

A method of reducing image brightness with tungsten filament lamps is to use a variable transformer, such as a Powerstat or Variac unit. This can reduce lamp intensity to a comfortable level for visual use or for intensity control when black-and-white films are used. Lamp life will be considerably prolonged also. If color films are to be exposed, the lamp must be used at the normal rated voltage for photography. Newer tungsten-halogen lamps must not be used below their rated voltage.

COLOR PHOTOMICROGRAPHY

FILMS

The ultimate function of the microscope and the illumination is to produce the best possible image of the specimen. If the specimen is colored, as with biological stains, then the image will also be colored, and can be recorded to best advantage on a color film. Filters are almost always necessary when a color film is to be exposed, principally for control of color balance.

A working knowledge of color film characteristics, as well as of the action of specific filters, is essential in color photomicrography if the micro-image is to be recorded efficiently.

KODAK Color Films for Use In Photomicrography

Eastman Kodak Company manufactures two general types of color films for exposure in a camera. They are designated as *reversal* color films and *negative* color films. The reversal films yield direct-positive color transparencies after reversal processing; the resultant colors are comparable to those seen in the original subject. Color transparencies are viewed either by projection or on a suitable illuminator. The negative films yield color negatives after processing; the colors in the recorded image are *complementary* to the corresponding colors in the subject. Color negatives must be printed onto a positive color material in order to obtain a reproduction of the original subject colors.

KODAK EKTACHROME Films, KODACHROME Films, and KODAK Photomicrography Color Film 2483 are reversal color films, whereas KODACOLOR-II Film and KODAK EKTACOLOR Professional Films are of the color negative type.

Color films are further classified with regard to the kind of illumination for which they are balanced in manufacture. This is either artificial light (tungsten) or daylight. A film intended for use with artificial light is usually specified as Type A, Tungsten, or Type B, indicating that the light source for which the film was balanced had a color temperature of 3400 K (Type A) or 3200 K (Tungsten and Type B). A film specified as "Daylight Type" is balanced for average sunlight at 5500 K. In addition, KODAK EKTACOLOR Professional Films are designated either Type L or Type S. The Type L film is balanced for tungsten light at 3200 K and for long exposure times (1/10 second to 60 seconds). The Type S film is balanced for daylight illumination and for short exposure times of 1/10 second or less.

When a particular film is to be exposed in photomicrography, and the illumination differs from that for which the film was balanced, specific filters must be placed in the light beam to adjust the illumination. KODAK Light Balancing Filters were made for this purpose. Failure to make this adjustment may result in erroneous color balance in the recorded image. (See KODAK Light Balancing Filters, page 35.)

All color films are rated according to their "speeds," or sensitivity to light. Film speeds are assigned by a standard rating system as numbers which are directly proportional to sensitivity—the larger the number, the faster the speed. A film rated at a speed of 160 is twice as fast in its reaction to light as another film rated at 80, four times as fast as a film at 40, etc. The film speeds discussed in this book are those specified by the American National Standards Institute. The speed number given to a film is commonly called the "ASA speed."

Table 4 contains a listing of Kodak color films for photomicrography, their speeds, how they are balanced, the various available sizes, and the process involved.

Selection of Color Films

The film characteristics to consider in selecting a color film for photomicrography are size, speed, and the ability of the film to record specimen colors as accurately as possible. Other factors are contrast, granularity, illumination, and color balance.

The film size, of course, is governed by the camera to be used. Most photomicrographic cameras accommodate 35 mm film with a 24 x 36 mm frame size. Others accommodate sheet films of specific size. The widest selection of color films is in 35 mm size, while the narrowest is in sheet films.

In photomicrography, 35 mm reversal color films are most often used. These films yield color slides commonly used in 2 x 2-inch slide projectors, such as KODAK CAROUSEL Projectors. When these films are processed, the individual frames are usually mounted either in cardboard or in glass mounts for direct insertion in the projector. The convenience of 35 mm slides is unquestioned—for handling, filing, and transporting.

The selection of a Kodak reversal color sheet film is as follows: Photomicrography Color Film 2483

(4 x 5-inch size only), intended for Process E-4; Kodak Ektachrome Film 6116, Type B (Process E-3), and Kodak Ektachrome Film 6115, Daylight Type (Process E-3). The latter two films are available in all standard sheet sizes. Projectors for sheet-film transparencies are not available from Eastman Kodak Company, but can be obtained from photographic dealers. A projector of this type usually handles a 3¼ x 4-inch glass slide, so any film larger than this size must be cut down to fit masks and cover glass.

Negative color films offer versatility with regard to the kinds of prints which can be made from color negatives. Positive color transparencies can be made from a color negative—either on Kodak Ektacolor Slide Film 5028 for 35 mm slides or on Kodak Ektacolor Print Film 4109 (Estar Thick Base) to make large transparencies in a variety of sizes. Color prints of many sizes can be made from a color negative by using Kodak Ektacolor 37RC Paper. This paper produces excellent color prints from Kodacolor II and Ektacolor negatives. Black-and-white prints can be made readily from color negatives by using Kodak Panalure Paper or other panchromatic paper. Panalure Paper is a fast panchromatic enlarging paper which renders colors in their correct gray tone. Kodacolor II Film can be obtained in 35 mm as well as in other roll sizes.

The high film speed is important when a specimen is in motion, or possibly when the illumination intensity is very low. In either case, the use of a high-speed film may be of advantage. However, micro-subjects normally are stationary and film speed is unimportant because exposure time can be controlled over a wide range.

TABLE 4

Kodak Color Films for Photomicrography

Film	Balance	ASA Speed*	Sizes	Process	Commercial Processing	Do-it-Yourself Processing	Kit Sizes
Kodak Photomicrography Color 2483	Daylight	16	PCF135 (36 exp) PCF415 (35mm x 125-ft) 4 x 5-inch sheet-film size	E-4	×	×	1-qt
Kodak High Speed Ektachrome (Daylight)	Daylight	160	EH135 (20 & 36 exp) EH120, EH126 (20 exp)	E-4	×	×	1-qt
Kodak High Speed Ektachrome (Tungsten)	3200 K	125	EHB135 (20 & 36 exp) EHB120	E-4	×	×	1-qt
Kodachrome 25	Daylight	25	KM828 KM135 (20 & 36 exp)	†	×	No Kits	—
Kodachrome II Professional, Type A	3400 K	40	KPA135 (36 exp only)	†	×	No Kits	—
Kodachrome 64	Daylight	64	KR135 (20 & 36 exp) KR126	†	×	No Kits	—
Kodak Ektachrome-X	Daylight	64	EX135 (20 & 36 exp) EX126 (20 exp), EX127, EX120 EX620, EX828	E-4	×	×	1-qt
Kodacolor II	Daylight	80	CX116, CX120, CX620 CX126, CX127, CX135-20 CX616, CX828	C-41	×	×	1-pint 1-gal
Kodak Ektachrome 6115, Daylight Type (Process E-3)	Daylight	50	All standard sheet-film sizes.	E-3	×	×	½-gal 1-gal
Kodak Ektachrome Professional, Daylight Type (Process E-3)	Daylight	50	EP120	E-3	×	×	½-gal 1-gal
Kodak Ektachrome 6116, Type B (Process E-3)	3200 K	32	All standard sheet-film sizes.	E-3	×	×	½-gal 1-gal
Kodak Ektacolor Professional 6102, Type L (Long Exposure)	3200 K	64 (5-second exposure)	All standard sheet-film sizes.	C-22	×	×	1-pint 1-gal
Kodak Ektacolor Professional, Type S (Short Exposure)	Daylight	100	CPS135 (36 exp) CPS120, CPS220 CPS620 and all standard sheet-film sizes.	C-22	×	×	1-pint 1-gal

*Film speeds quoted depend upon illumination level and exposure time, and refer to use of films under recommended conditions.
†Processed by Kodak or laboratory offering such service.

Normally, color films are not produced with a wide selection of either contrast or granularity. Recently, a new film, KODAK Photomicrography Color Film 2483, was introduced. It features the highest contrast and highest resolving power of any Kodak color reversal film used for photomicrography. (Data on this film is available on request. See page 3 for details.) Of the other Kodak color reversal films, KODACHROME 64 and EKTACHROME-X Films exhibit the most contrast, although their contrasts are only slightly higher than the other color films. KODACHROME 25 Film exhibits finer granularity than either KODACHROME 64 Film or EKTACHROME-X Film. These characteristics have been established in manufacture. Any attempts to change contrast, granularity, or speed by departures from recommended processing may result in an undesirable change in color balance. KODAK EKTACHROME Films designed for Process E-4, however, do have some leeway in this procedure.

As previously stated, color films are made for use with either daylight or artificial light. The choice here is optional, since either type can be used successfully in photomicrography if the illumination is properly adjusted with light-balancing filters. Normally, however, if tungsten illumination is used with the microscope, a color film balanced for artificial light should also be used. Daylight-type films are suggested when the light source has daylight quality. Such sources as the xenon arc or electronic flash give this illumination. Color films balanced for daylight can also be used with tungsten sources if the illumination is correctly adjusted. The use of daylight films extends the selection of films available when one is looking for a film which will record and reproduce specimen colors most accurately. The rendition of specimen color is related to the sensitivity and spectral response of the color film.

Rendition of Stain Color

The many biological stains used in specimen preparation, such as tissue sections and smears, represent all of the colors of the visible spectrum. Color films, however, show considerable difference in sensitivity and response to various colors. For this reason, the color of one stain may record better on one color film than on another. It depends on where *that* color appears in the spectrum and how sensitive the color film is to *that* region of the spectrum. The rendition of a particular color also depends on the particular dyes formed in the emulsion layers of the film; different color films utilize different dye systems. So, in photomicrography, specimen dye-colors are recorded on a color film containing different dyes. Unfortu-

TABLE 5

EASTMAN Biological Stains*

Catalog Number	Chemical Name	Field of Use				
		Animal Histology	Plant Histology	Cytology	Pathology-Bacteriology	Fluorescent Microscopy
C1740	Acid Fuchsin	✓	✓	✓	✓	✓
C1051	Alizarin Red S	✓		✓	✓	
C1741	Aniline Blue W.S.	✓	✓		✓	
C8688	Auramine O	✓	✓			✓
C8680	Azocarmine G	✓				
C8689	Azure A	✓			✓	
C8683	Azure B				✓	
C8693	Azure C	✓			✓	
C1762	Basic Fuchsin	✓	✓	✓	✓	
C1742	Bismarck Brown Y	✓	✓		✓	
C1743	Brilliant Cresyl Blue	✓		✓		
C624	Brilliant Green				✓	
C1745	Carmine Alum Lake	✓	✓	✓	✓	
C8684	Chlorazol Black E	✓				
C770	Congo Red		✓		✓	
C8687	Cresyl Violet Acetate	✓			✓	
C1350	Crystal Violet	✓	✓	✓	✓	
C5322	Eosin B	✓			✓	
C1746	Eosin Yellowish	✓	✓	✓	✓	
C110	Erythrosin B	✓	✓		✓	
C8702	Ethyl Eosin	✓			✓	
C8686	Fast Green FCF	✓	✓	✓		
C8685	Giemsa Stain			✓	✓	
C309	Hematoxylin	✓	✓	✓	✓	
C1009	Indigo Carmine	✓				
C4444	Janus Green B	✓		✓		
C8705	Jenner's Stain			✓		
C5911	Light Green SF, Yellowish	✓	✓	✓	✓	
C1264	Malachite Green	✓	✓	✓	✓	
C1979	Martius Yellow		✓		✓	
C573	Methylene Blue	✓	✓	✓	✓	
C8913	Methylene Violet (Bernthsen)				✓	
C1767	Methyl Green		✓	✓	✓	
C432	Methyl Orange	✓	✓	✓	✓	
C1309	Methyl Violet 2B	✓	✓	✓	✓	
C725	Neutral Red	✓			✓	
C3536	Nigrosine W.S.	✓	✓		✓	
C8679	Nile Blue A	✓			✓	
C1751	Orange G	✓	✓	✓	✓	
C196	Orange II	✓	✓			
C1294	Orcein	✓		✓	✓	
C8681	Phloxine B	✓	✓		✓	
C1752	Pyronin B				✓	
C8707	Pyronin Y				✓	
C2245	Rose Bengal				✓	✓
C1753	Safranin O (Reddish)	✓	✓	✓	✓	
C1754	Sudan III	✓				
C1273	Sudan IV	✓	✓		✓	
C8690	Sudan Black B	✓			✓	
C8709	Tetrachrome Stain (MacNeal)			✓	✓	
C1755	Thionin	✓	✓	✓	✓	
C1756	Toluidine Blue O	✓		✓	✓	
C8682	Wright's Stain			✓	✓	

*Available in small quantities from suppliers of EASTMAN Organic Chemicals or directly from Eastman Kodak Company.

nately, the results are not always satisfactory.

There is no *one* color film which will record all specimen colors accurately. If specimen colors must be reproduced with reasonable fidelity, it may be necessary to experiment with different films to find one which is most suitable.

Although a comprehensive survey has not been made on the rendition of all biological-stain colors on the different color films, there are some common stains whose color rendition is known. Probably the most common stain is one called "eosin." This reddish stain is widely used with tissue sections in animal histology in combination with another stain known as "hematoxylin," and in combination with "methylene blue" stain for blood smears (Wright's Stain). This stain color reproduces well on KODAK Photomicrography Color Film 2483 but not on KODACHROME Films, especially when the stain is very light in color. The use of a *didymium* glass filter enhances its portrayal with KODACHROME Films. KODAK EKTACHROME-X Film does show excellent rendition of eosin color without this special filter. Filtering (see next section) can often yield an improvement in the representation of many other stains.

In some neurological techniques a silver preparation is often used. Tissue and nerve cells appear deep yellow as shown on EKTACHROME-X Film in Plate IV, C, page 40.

Processing Color Films

After color films have been exposed in a camera, they must be properly processed, either by the user or by a commercial processing laboratory. Eastman Kodak Company provides processing service for KODACHROME Films and for KODAK EKTACHROME Films *in rolls*. KODACOLOR II Film, also in rolls, is both processed and printed by Kodak. Sheet color films, however, are *not* processed by Kodak.

Many commercial laboratories are equipped to process and print *all* Kodak color films. Some of these processing laboratories offer specialized services, such as fast service enlargements with cropping, etc., which will be helpful to the photomicrographer.

Color films to be processed by Kodak laboratories should be sent either through photographic dealers or in special mailers directed to Kodak. KODAK Prepaid Processing Mailers can be purchased from dealers. When mailers are used, processed films will be returned directly to the customer.

Some color films can be processed by the user. They include KODAK Photomicrography Color Film 2483, EKTACHROME Films (roll or sheet), KODACOLOR II Film, and EKTACOLOR Professional Films. (See Table 4, page 32.) Chemicals are available in kit form for each color process. Complete information is given in data sheets about mixing chemicals, processing times, agitation, and temperature. Further information is available in Kodak Publication No. R-19, KODAK *Color* DATAGUIDE, available from your photo dealer.

KODACHROME Films *cannot* be processed by the user. They must be processed by a facility that has suitable equipment for this purpose.

Increasing Color-Film Speed

It is possible to use either KODAK EKTACHROME-X or KODAK High Speed EKTACHROME Films at film-speed ratings higher than normal by increasing the first-developer time a specific amount. These films can be processed with KODAK EKTACHROME Film Chemicals, Process E-4. Increasing film speed by altering processing is not possible with any other Kodak color films.

Best color quality will be obtained when either of the above films is processed normally. If a compromise in color quality is acceptable, however, the increased speed may be an advantage in some applications.

Processing for increased speed can easily be accomplished when the user develops his own color film. If film is usually sent to a commercial laboratory, the user should check with the laboratory to find out if this service is available.

Table 6 gives the suggested changes in first-developer times, Process E-4, and the resultant speed ratings for various EKTACHROME Films.

TABLE 6

Increasing Color Film Speed

First-Developer Time	KODAK High Speed EKTACHROME Film (Daylight)	KODAK High Speed EKTACHROME Film (Tungsten)	KODAK EKTACHROME-X Film
Normal*	160	125	64
Normal plus 35% increase	320	250	125
Normal plus 75% increase	640	500	250

*Refer to instruction sheet packaged with Process E-4 chemicals for normal first-developer time.

Kodak Processing Service for Increased Speed

A KODAK Special Processing Envelope, ESP-1 (available from photographic dealers), enables a customer to send KODAK High Speed EKTACHROME Film (Daylight or Tungsten) to a Kodak laboratory in the United States for special processing for increased speed. The Daylight film will be processed for an ASA speed of 400; the Tungsten film, for an ASA speed of 320. The cost of the special envelope prepays the extra charge for this service. This special service is available only for the 135-20, 135-36, and 120 sizes of KODAK High Speed EKTACHROME Film.

FILTERS

The ultimate function of both the microscope and the illumination is to produce the best possible image of a specimen. A photomicrograph is a record of this image on a photographic, or light-sensitive, material. If the specimen is colored, as with biological stains, then the image will also be colored and will be recorded ideally on a color film. If a colored image is to be recorded on a black-and-white material, the colors must be reproduced as tones of gray that satisfactorily represent the color brightnesses in the specimen. It is usually necessary to use specific light filters either to provide correct rendition of colors on color film or to record the colors as appropriate gray tones with suitable contrast on a regular film or plate.

KODAK Light Balancing Filters

These filters are intended for the adjustment of illumination when it differs from that for which a color film is balanced. Illumination color quality is usually expressed as "color temperature" in degrees on the Kelvin scale. KODACHROME II Professional Film (Type A) is balanced in manufacture for a light source having a color temperature of 3400 K. If the light source to be used in exposure of this film has a *lower* color temperature than 3400 K, its illumination can be adjusted with one or more KODAK Light Balancing Filters of the 82 series to approximate a 3400 K color temperature. There are four filters in this series: No. 82, 82A, 82B, and 82C. Each will effectively raise color temperature by definite increments. Of course, these filters don't *actually* change the color temperature of the light source, but they do modify the illumination to simulate a higher color temperature. The No. 82 filter will effectively increase the color temperature by 100 K; the No. 82A, by 200 K; the No. 82B, by 300 K; and the No. 82C, by 400 K. If the light source had a color temperature of 3000 K, therefore, its illumination could be adjusted to 3400 K with a No. 82C filter. The above filters can be used in combination to adjust illumination by greater amounts than 400 K. For example, if the light source were 2800 K, its illumination could be adjusted to 3400 K with a No. 82C plus a No. 82A filter. All of the 82 series filters are light blue in color.

Another group of KODAK Light Balancing Filters applies when color temperature is too high for a film. This is the No. 81 series, which includes No. 81, 81A, 81B, 81C, 81D, and 81EF. These filters are yellowish in color and will effectively *decrease* color temperature by 100 K, 200 K, 300 K, 400 K, 500 K, and 600 K. These figures are only approximate, however, since the actual amount depends upon the initial color temperature. The No. 81 series filters are not often used in photomicrography, however, since too high a color temperature seldom occurs.

An important fact to be considered is that the actual color temperature of a light source may be unknown. Then, too, the illumination quality at the film plane may be different than that emitted from the source because of optical absorptions within the microscope and the illumination system. Most built-in illumination systems, for example, contain a diffuse surface, which will effectively *decrease* color temperature by about 200 K. So, even if the source were known to have a specific color temperature, the illumination color-quality supplied to the film might be different. Most often, it is *lower* in effective color temperature than is indicated by the source.

Tungsten-filament lamps are often used in photomicrography, particularly in the built-in illumination systems, where 6-volt coil-filament lamps are common. These lamps, manufactured by several firms, differ in color temperature through a range of about 2800 K to 3200 K, even when used at the specified voltage. A lamp in one microscope system, then, may differ in color temperature from the lamp in another system. It is difficult to say, then, which light-balancing filter, or filters, should be used.

The most practical method for determining the correct filters is to make color-balance tests, using different filters or combinations of filters. The 82 series of light-balancing filters usually applies with tungsten illumination, since the color temperature is almost invariably too low for an artificial-light film. If the initial color temperature of the source is unknown, a series of exposures might be made using the No. 82, 82A, 82B, 82C, 82C + 82, and 82C + 82A filters and a reversal color film balanced for artificial illumination. After the color film is processed, the different exposures could be examined on an illuminator to determine which one showed the most pleasing rendition of specimen colors and a clean, almost-white background. The filter or filters used to make that exposure would then be correct for future exposures. If a transformer was used to provide the 6 volts to the lamp, the same setting should always apply for making future photomicrographs. Several voltage settings are often available on a transformer. The *highest* setting should normally be utilized, since the highest color temperature for the lamp is obtained at this setting.

If the color temperature of the source is known, even approximately, the color-balance test series can be much shorter; very often, only about two filters or combinations need to be applied.

Light-balancing filters should be placed between the illuminator and the condenser of the microscope. Separate illuminators usually contain filter receptacles for the purpose.

TABLE 7

Kodak Filters* for Kodak Color Films

Light Source	Tungsten, Type B, or Type L Film (3200 K)	Type A Film (3400 K)	Daylight-Type Film
	Filter No.	Filter No.	Filter No.
6-Volt Ribbon-Filament (Average C.T. = 3000 K)	82A	82C	80A + 82A
6-Volt Coil-Filament (Average C.T. = 3100 K)	82	82B	80A + 82
100-Watt Coil-Filament (Average C.T. = 3100 K)	82	82B	80A + 82
300- to 750-Watt Coil-Filament (3200 K)	None	82A	80A
Zirconium Arc (3200 K)	None†	82A	80A
Photoflood Lamp (3400 K)	81A	None	80B
Carbon Arc (3700 K)	81D†	81B	80C
Xenon Arc (5500 K)	85B	85	None†

*The filters suggested are considered to give approximate color-temperature compensation. Color test exposures are often necessary to obtain best color balance.

†A No. 2B filter is often used to absorb unwanted ultraviolet from arc illumination.

Table 7 contains average filter recommendations for different light sources and various color films. (See also the nomograph on the inside front cover.) These recommendations can only be considered as approximate, because of the variance in the color temperature of light sources and the electrical conditions at the time of exposure. Voltage regulators are desirable for critical work.

Kodak Light Balancing Filters can be obtained from photo dealers. They are supplied as gelatin film squares (50, 75, 100, or 125 mm). These gelatin film squares can be inserted into two-part pressed metal frames called Kodak Gelatin Filter Frames, which make the filters convenient to handle and store. These frames are also available through photo dealers. If glass-mounted filters are needed, they can be obtained from suppliers such as Tiffen Optical Company, 71 Jane Street, Roslyn Heights, New York 11577.

All daylight-type color films can be used in photomicrography, regardless of the type of illumination, if the correct filters are placed in the *light beam* to adjust the illumination to daylight quality. These films can also be used, of course, with light sources of daylight quality—such as the xenon arc or electronic flash—and probably without any light-balancing filters. With tungsten lamps and the zirconium arc, however, the use of specific light-balancing filters is necessary if daylight-type films are to be exposed.

Kodak Wratten Gelatin Filter, No. 80A will adjust illumination color-quality from 3200 K to daylight. If color temperature is below 3200 K, as it often is, one or more of the 82 series filters will also be needed. In order to establish which filter of the 82 series should be used with the No. 80A filter, a color test-exposure series can be made. Five exposures will probably suffice; one with the No. 80A filter alone and four with the combinations of No. 80A + No. 82, No. 80A + No. 82A, No. 80A + 82B, and No. 80A + No. 82C. One of the resultant exposures will show the best color balance. The correct combination should be used in subsequent photomicrography when a daylight-type film is exposed.

KODAK Color Compensating Filters

Undesirable color effects are caused by several factors in color photomicrography in addition to the one already mentioned—exposing a color film to illumination other than that for which the film was designed. These effects can usually be corrected with Kodak Color Compensating Filters—available in various densities in red, green, blue, cyan, magenta, and yellow. These filters are commonly called "CC Filters" and can be obtained through photographic dealers. They are supplied as gelatin film squares and can be inserted in Kodak Gelatin Filter Frames.

The various general effects that can be corrected with CC Filters are discussed in "Factors Affecting Color Balance," page 41. Pale color compensating filters can sometimes be used to enhance the rendition of certain stains. However, the benefits gained by use of a filter of the approximate color of the stain must be balanced against a possible degradation of complimentary stain color.

Neutral Density Filters

Neutral density filters can be used in photomicrography to *reduce* image brightness as a means of controlling exposure time. A neutral filter will absorb a specific amount of light, depending on its *density,* without affecting the color quality of the illumination. The principal application of such filters is in the exposure of color films. They can also be used with black-and-white films to prevent overexposure.

If a very intense light source is used to provide illumination, the correct exposure time (as determined by a test-exposure series or by light measurement) may be shorter than the fastest available shutter speed. A neutral density filter can be placed in the light beam to reduce image brightness so that the exposure time will be within the shutter range.

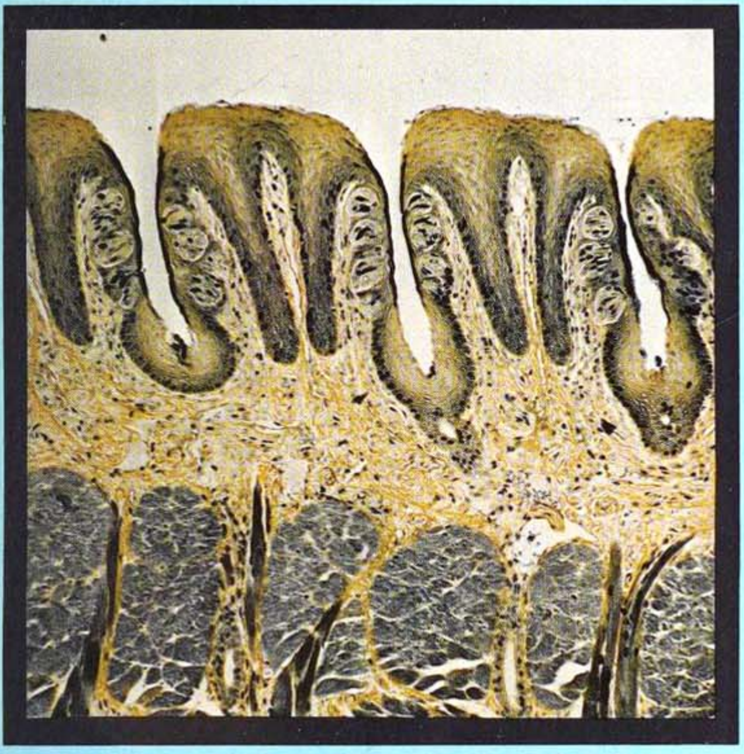


Plate I, A—Taste buds in rabbit tongue. × 100. Iron hematoxylin stain. KODAK EKTACHROME Film, Type B.

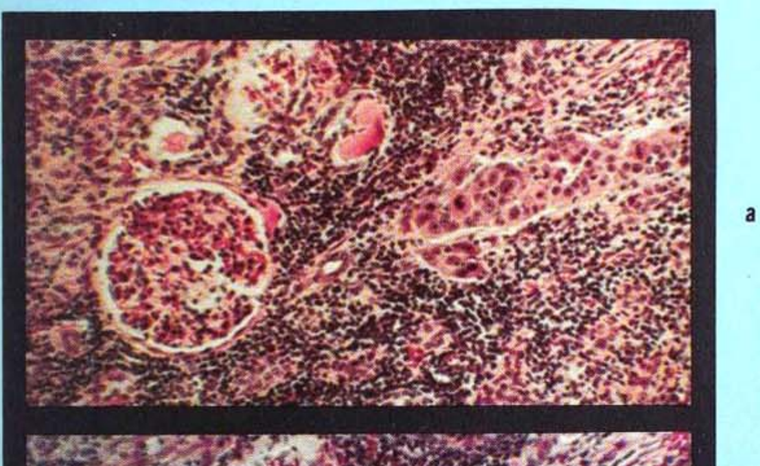


Plate I, B—Photomicrographs on KODACHROME II Film, Daylight Type

A. Uncorrected tungsten illumination
B. Illumination adjusted with KODAK Light Balancing Filters (No. 80A and 82A)

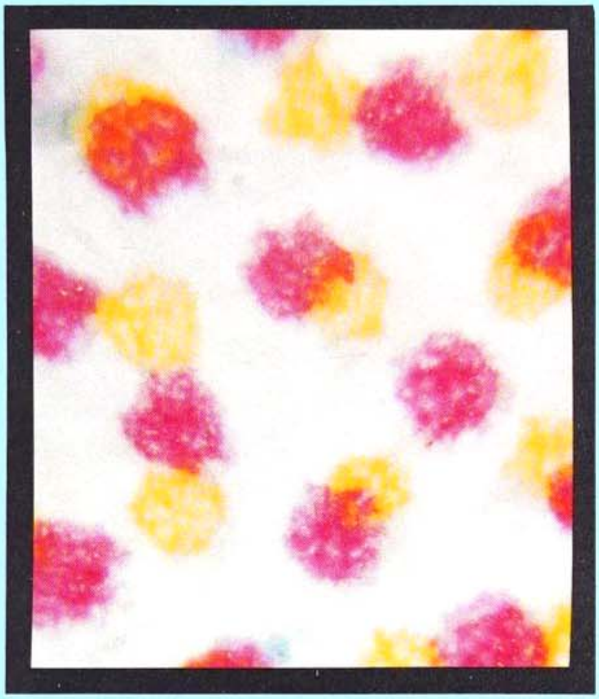


Plate I, C—Highlight dots on printed material— × 100. KODAK EKTACHROME Film, Type B.

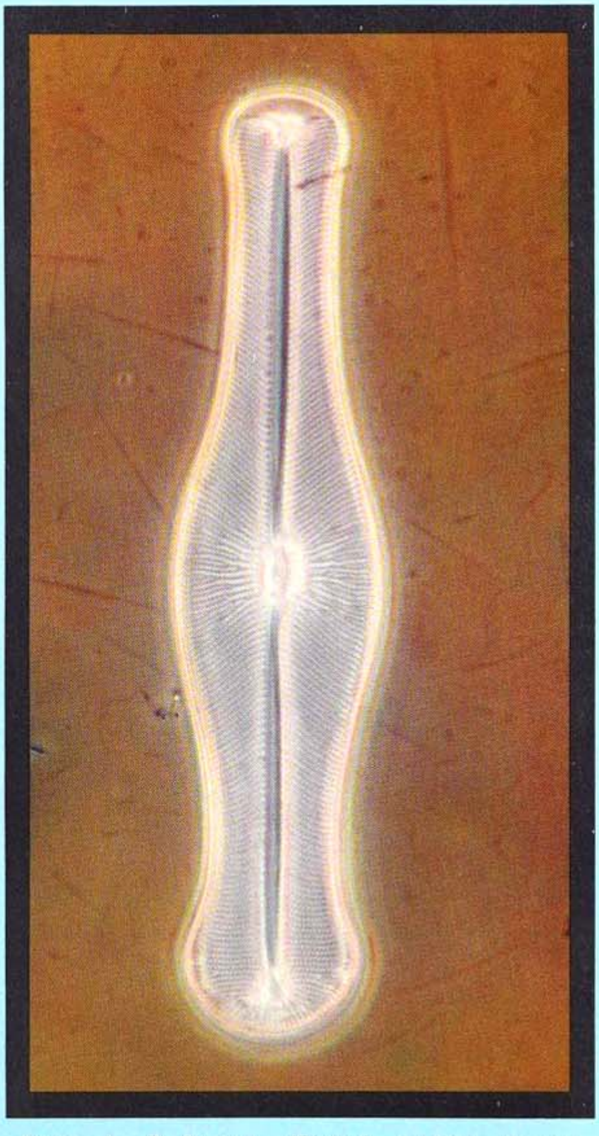


Plate I, D—Diatom (Phase)— × 1000. KODAK EKTACHROME Film, Type B.

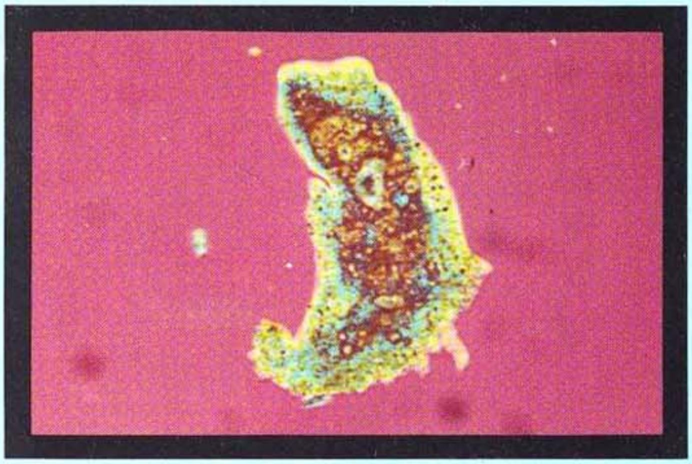


Plate II, A—Amoeba proteus (interference microscope) —× 150.

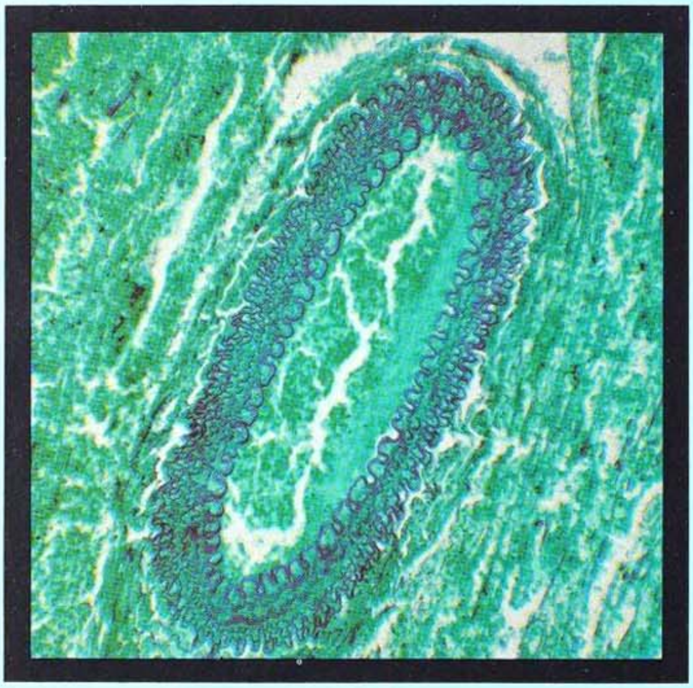


Plate II, C—Micro-artery— × 125. Aldehyde fuchsin and light green stains. KODAK EKTACHROME Film 6116, Type B (Process E-3.)

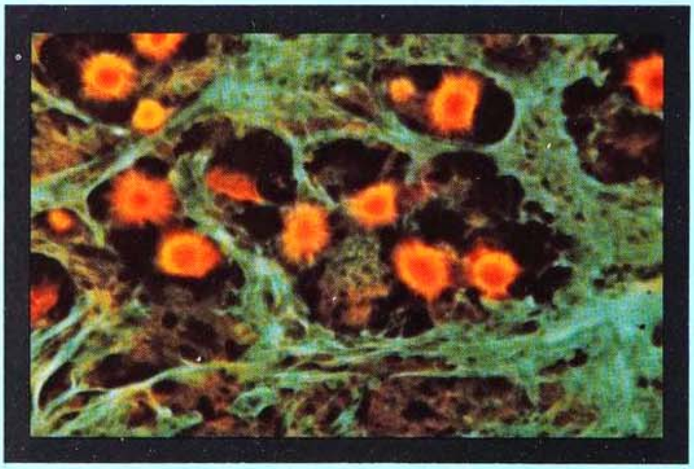


Plate II, D—Fluorescence photomicrograph. Acridine orange stain. KODAK High Speed EKTACHROME Film (Daylight).

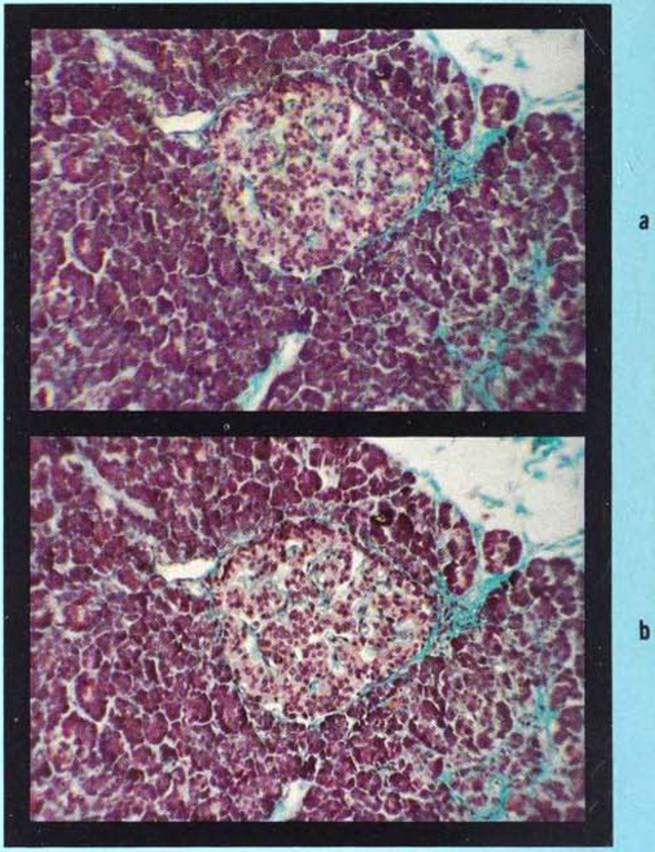


Plate II, B—Human pancreas, × 150. Comparison of image quality with (a) apochromat, (b) achromat.

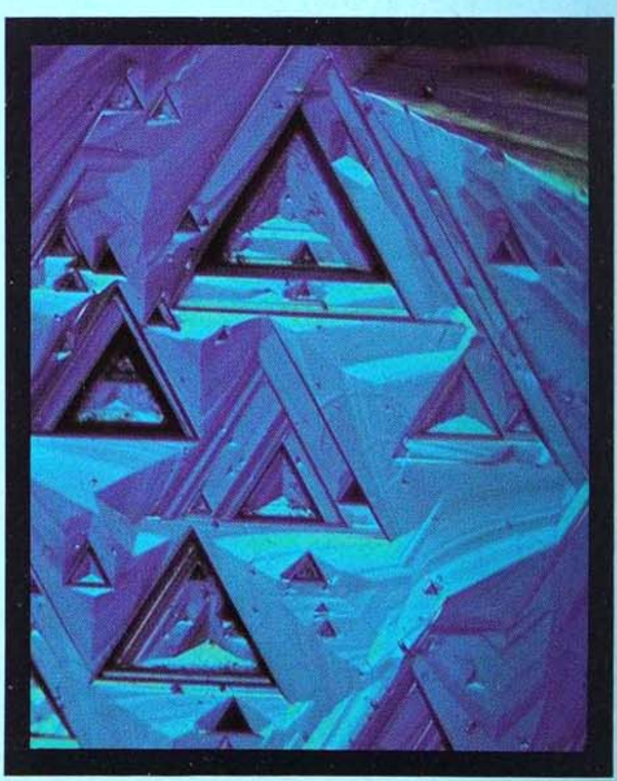


Plate II, E—Unpolished surface of an industrial diamond photographed by the Francon system. This method is similar to the Nomarski system (discussed under "Specialized Techniques and Applications"), but it produces interference from surface light reflected by thick or opaque specimens instead of from transmitted light.

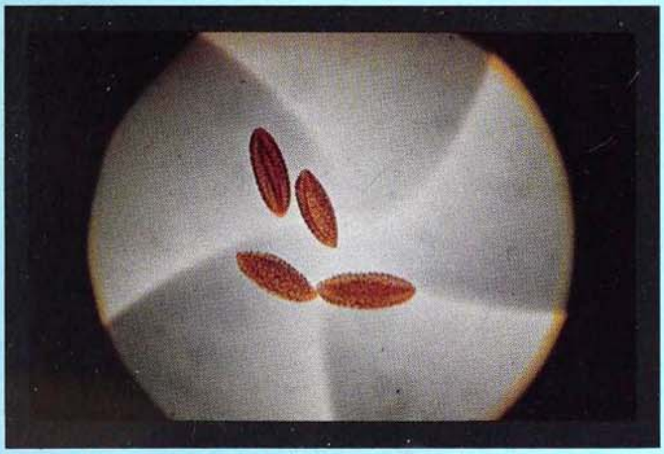


Plate III, A—Image of shutter blades—shutter too far from eyepoint.

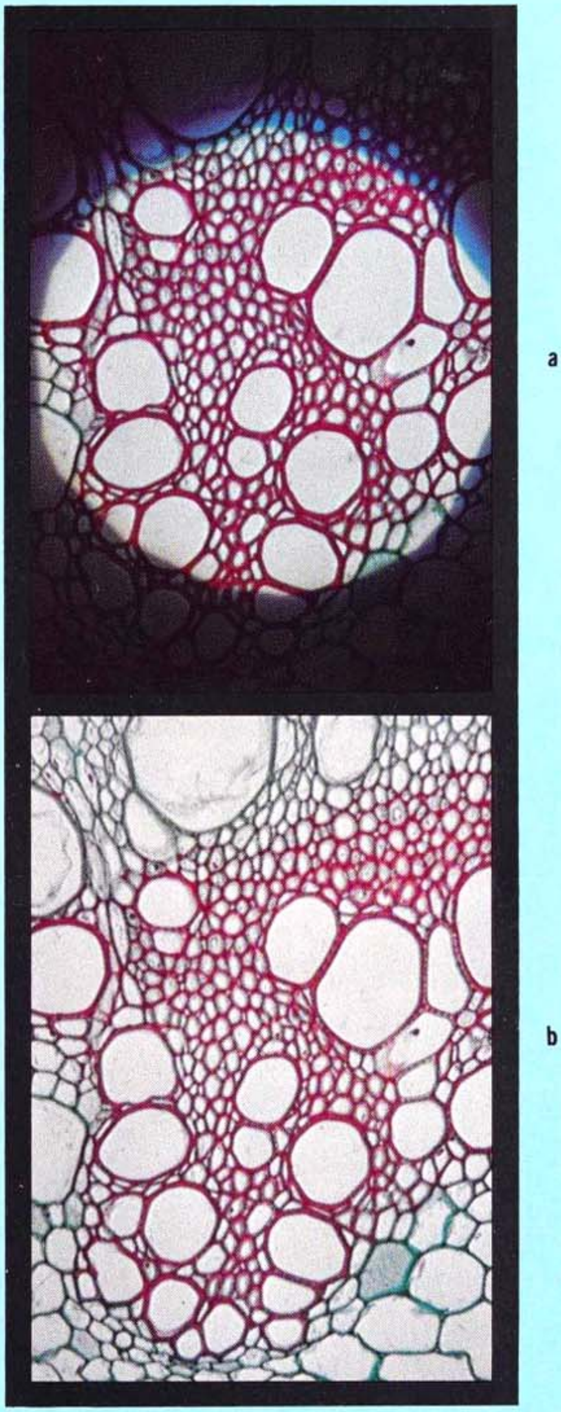


Plate III, B—(a) Image of field diaphragm; (b) Image removed by opening field diaphragm.

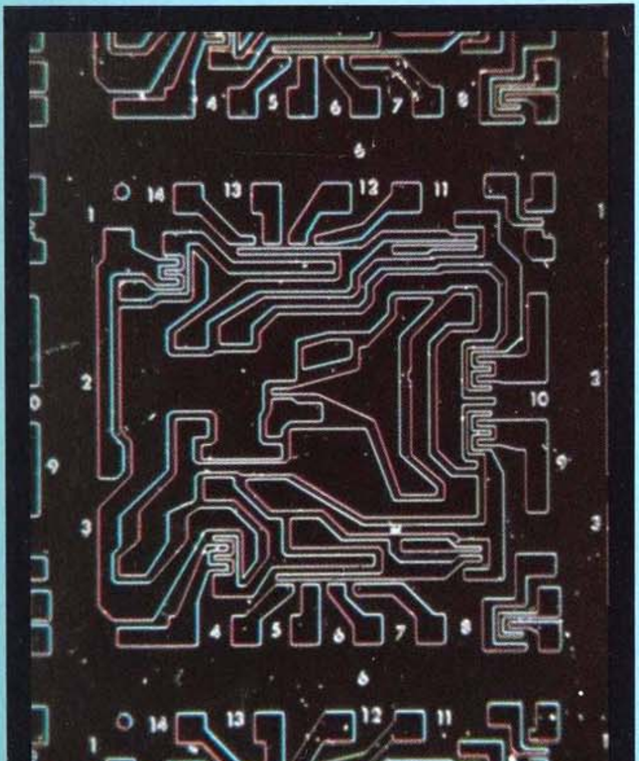


Plate III, C—Printed micro-circuit— × 40. Reflected light.

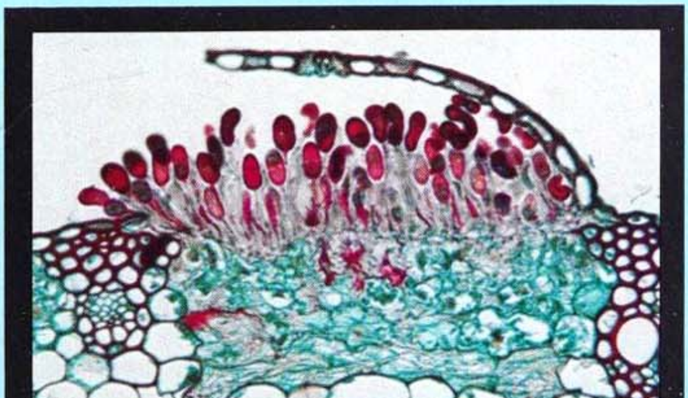


Plate III, D—Black stem rust of wheat.

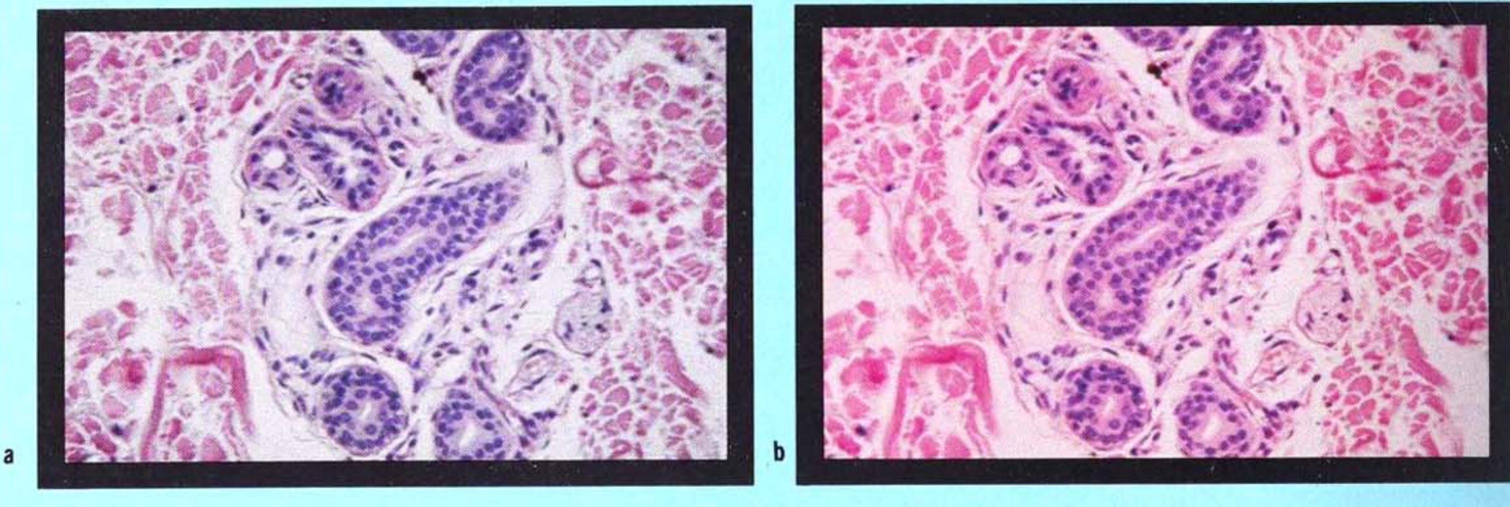


Plate IV, A—Effect of eosin stain (a) without didymium filter; (b) with didymium filter.

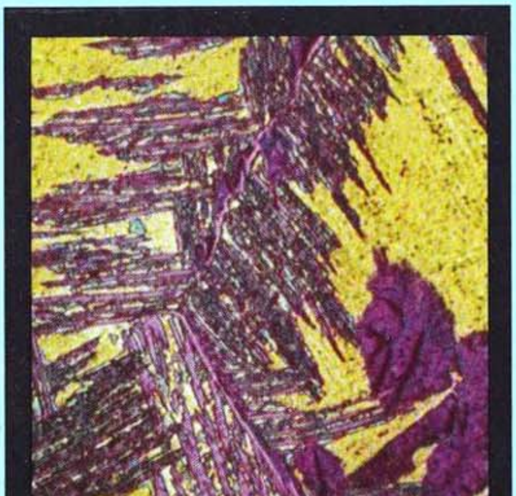


Plate IV, B—Zinc-silver alloy, chemical stain.

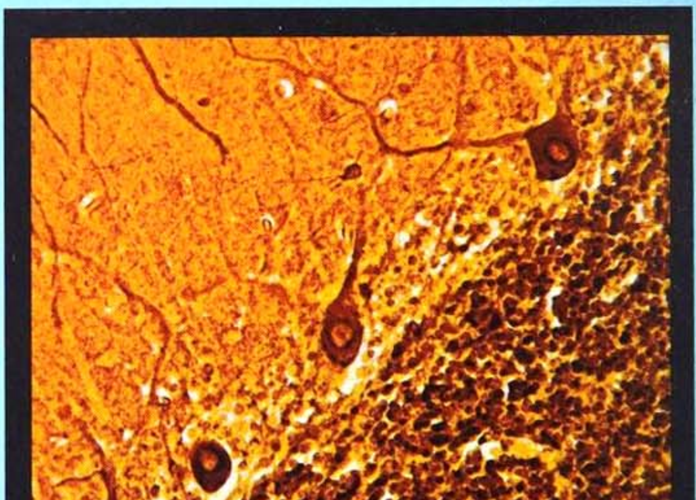


Plate IV, C—Nerve cells in human cerebellum—×200, Silver impregnation. KODAK EKTACHROME Film, Type B.

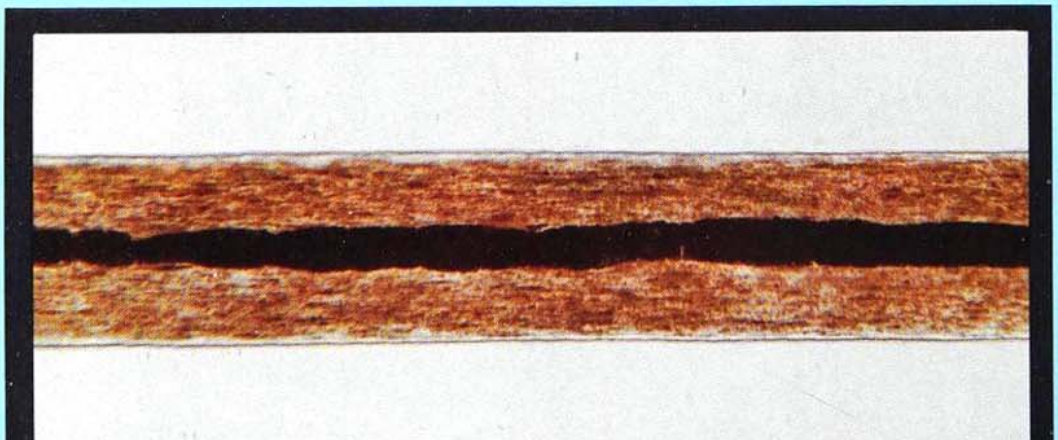


Plate IV, D—Human hair, brown—×150. KODAK EKTACHROME Film, Type B.

The absorption of light by a neutral filter is directly proportional to the filter's *density*. The greater the density, the greater the amount of light absorbed. Also, the greater the density, the lesser the amount of light *transmitted*. Density is defined mathematically as the logarithm of the reciprocal of transmittance ($D = log\frac{1}{T}$). Kodak Wratten Neutral Density Filter, No. 96, is available in a wide range of densities from 0.10 to 4.0. The amount of light transmitted by these neutral density filters varies from 80 percent to 0.01 percent, respectively. The following table includes various filter densities available and their corresponding transmittances.

Density	Percent Transmittance	Density	Percent Transmittance
0.10	80.0	0.80	16.0
0.20	63.0	*0.90	13.0
*0.30	50.0	1.00	10.0
0.40	40.0	2.00	1.0
0.50	32.0	3.00	0.10
*0.60	25.0	4.00	0.010
0.70	20.0		

*Most often used in photography.

The netural density filters most often used in photography to control exposure time have densities of 0.30, 0.60, and 0.90. As shown in the table, these filters have transmittances of 50, 25, and 13 percent. Since a density of 0.30 has a transmittance of 50 percent, it can be used to reduce brightness by a factor of 2. A density of 0.60 has a transmittance of 25 percent and a reduction factor of 4. These densities can be used in combination because the total density equals the sum of the individual densities.

Here is a sample exposure calculation involving neutral density filters: Suppose that a very bright light source provides illumination, and that a reasonably fast film is to be exposed. The correct exposure time is determined to be 1/125 second, but the fastest available shutter speed is only 1/60 second. This shutter speed would cause overexposure. A density of 0.30 could be placed in the light beam to reduce image brightness by 50 percent; 1/60 second would then be the correct exposure. If a density of 0.60 were placed in the light beam, the correct exposure setting would then be 1/30 second.

Because reversal color films have a very short exposure latitude, it sometimes happens that the best exposure is considered to be *between* two shutter speeds. For example, 1/60 second may be too short, causing slight *underexposure;* but 1/30 second may be too long, causing slight *overexposure*. In this case, a neutral density of either 0.10 or 0.20 could be used to reduce the light level by less than a factor of 2; 1/30 second would then be the correct shutter speed.

Neutral density filters are also used to reduce visual image brightness. A very dense filter is placed in the light path when image brightness is too high for comfortable viewing. A density of 1.00 or more will usually suffice for this purpose, but it should be removed for photography because it may prolong exposure time too much. Long exposures should be avoided, when possible, because of "reciprocity effects" with color films and the resultant possibility of color shift and decreased film speed. (See "Factors Affecting Color Balance," below.)

Kodak Wratten Neutral Density Filter, No. 96, can be obtained from photographic dealers as gelatin film squares in 50, 75, or 100 mm. sizes on 0.1 mm gelatin with protective lacquer coating. Larger special sizes can be supplied also.

FACTORS AFFECTING COLOR BALANCE

At one time or another probably everyone who has made color photomicrographs has encountered undesirable color effects. Sometimes the reason is immediately obvious or can be easily determined by reviewing the conditions that affect exposure. Occasionally, however, the cause of erroneous color balance baffles even the most careful photomicrographer. If the cause *is* known, suitable correction can often be made in subsequent exposures; if it remains an unsolvable puzzle, a complete review of possible causes is indicated. Here are some suggestions.

Many undesirable effects can be neutralized by placing appropriate Kodak Color Compensating (CC) Filters in the microscope light beam. Some effects on color balance, however, are due either to incorrect use of optics or to problems related to film storage and film processing. These effects cannot be compensated with filters. The only remedy is to follow recommended procedures.

The most common reasons for a color-balance shift are listed in this section. Some are peculiar to photomicrography, but many can be encountered in any type of photography in which color film is used. Only color reversal films are considered here, since color negatives may not show a color change until they are printed. When color negatives *are* slightly off-balance, the effects can usually be corrected in printing if they are not too pronounced.

Table 9, page 46, summarizes some of the difficulties which may be encountered, their possible causes, and their remedies.

Color-Temperature Variance

Variation in color temperature is probably the most common reason for unexpected color shifts in color photomicrography. Whenever the illumination differs from that for which a particular film is balanced, the photomicrograph will have shifted color. If the color temperature of the light source is too high, an overall cold, bluish effect will be noticed in the photomicrograph. If the color temperature is too low, the photomicrograph will be too "warm" and will be either yellowish or reddish-yellow in appearance. The degree of color shift will vary according to the amount of color-temperature difference between the actual light source and that for which the film was balanced. An example of a large color shift is a micrograph made on daylight color film with uncorrected tungsten illumination. (See Plate I-B, page 37.) Color-temperature variance can be corrected by placing appropriate KODAK Light Balancing Filters in the illumination beam.

Heat-Absorbing Filters

Many light sources—such as high-wattage tungsten lamps and xenon arcs—emit a considerable amount of infrared in their illumination. This infrared is evidenced as heat and should be removed by an appropriate "heat-absorbing" filter to protect the microscope optics, the specimen, and any filters in the light beam. Some microscope illuminators have heat-absorbing glass filters built in. Unfortunately, the owner of such an illuminator may be unaware of the presence of the filter, which is usually colored green or blue-green. This coloration can affect the color balance of a photomicrograph. The transparency may appear too green or blue-green. This effect can be corrected in subsequent exposures by placing a "neutralizing" KODAK Color Compensating Filter in the light beam. A CC Filter of a complementary color is necessary. If the heat filter is greenish, a pale magenta CC Filter will absorb the green and produce a neutral effect. If the filter is blue-green, a pale red CC Filter is indicated. Heat-absorbing glass filters vary in their degree of coloration; it is not possible, therefore, to assign one specific CC Filter for correction. The correct filter must usually be determined by test exposures. If the heat filter can be removed from the lamp temporarily, you can place it on a transparency illuminator and view it through CC Filters of an appropriate complementary color until one is found which best neutralizes the heat-filter color. Of course, permanent removal of the heat filter also solves the problem, but this may not be wise, since permanent damage to the optics, filters, and specimen may result.

In some types of photomicrography, colored-liquid filters are used in the light beam to remove heat. In black-and-white metallography, for example, a dilute solution of copper sulfate in an absorption cell is often used. The blue-green color of the solution would dominate the color of a photomicrograph made on color film. During photography, therefore, the solution should be replaced by a cell containing distilled water.

Ultraviolet Radiation

Color film is very sensitive to ultraviolet radiation, which can be recorded as "blue" by the blue-sensitive emulsion layer. If ultraviolet is present, as in xenon-arc illumination, it may cause a color photomicrograph to appear too blue. An ultraviolet-absorbing filter, such as KODAK WRATTEN Filter No. 2B, should be used. The No. 2B Filter will absorb ultraviolet but will transmit all visible colors. The bluish effect of ultraviolet radiation on color film is often encountered in the photomicrography of metals. Most electronic flashtubes also emit ultraviolet. If a light source which emits ultraviolet is used in photomicrography with color films, the No. 2B Filter is recommended.

Neutral Density Filters

Some "neutral" filters normally used to reduce brightness may have a slight yellowish color, either inherently or as a result of aging. This yellowish color can be neutralized with a pale-blue color-compensating filter, either a CC05B or a CC10B. The effect generally isn't very great and can often be ignored.

Biological Stains

The stains used in coloring a section or smear to produce contrast between the elements of the specimen have individual properties of absorption and transmittance. Some stain colors reproduce very well when recorded on a specific color film, but others often appear quite different on film than when seen in the microscope. Eosin and fuchsin, for example, do not record well on KODACHROME Film. Their rendition, however, can be noticeably enhanced when a glass didymium filter is placed in the illumination beam. The thickness of the didymium filter should not be greater than 2 mm. If it is greater, a background color may appear or other stain colors may be degraded. Didymium glass filters are not available from Eastman Kodak Company but are available from the Corning Glass Works in Corning, New York 14830. If you order from Corning, specify half-standard thickness.

Both eosin and fuchsin stains record very well on

Photomicrography Color Film 2483 and fairly well on KODAK EKTACHROME-X Film without this special didymium filter. These films, however, are balanced for daylight. If a tungsten light source is used, appropriate light-balancing filters must be placed in the light beam to adjust the illumination to daylight quality.

Remember, because of the inherent deficiencies of the dyes used in color film, it may be impossible to record accurately all colors of the specimen.

Mounting Media

After a specimen has been stained, a drop of mounting medium is placed over it and a cover glass pressed into position. The mounting medium may be colored or it may become colored with age. In either case, an effect on color balance will be produced in the recorded image. This effect can be neutralized with a pale CC Filter of complementary color.

Canada balsam, for example, tends to become yellow with age. The degree of yellow depends on the thickness of the mounting medium and on its age. A blue CC Filter will compensate this yellow color.

However, when a considerable amount of Canada balsam has been used, such as with some whole mounts, and has become deeply colored from age, it may be impossible to neutralize the yellow color and still obtain good color balance for the specimen itself.

If an existing photomicrograph is of improper color because the mounting medium was colored and yet this picture must be used, a pale CC Filter of complementary color *could* be mounted with the photomicrograph for projection. This technique is practical if the discoloration is not too great.

Some mounting media remain colorless and should be used if possible. Table 3, on page 24, includes a general list of mounting media.

Chromatic Aberration

A very pronounced undesirable color effect which many people might have difficulty in tracing is a color variance introduced by chromatic aberration in the substage condenser. Both the Abbé and the aplanatic condenser have no correction for chromatic aberration. If they are not properly adjusted according to the technique of Kohler illumination, an undesirable color effect can be introduced.

When an Abbé condenser has been adjusted correctly to obtain a sharp image of the field diaphragm, that image will usually appear blue. (See the top of the circle in Plate III-B, page 39. If the image is not sharp, it will be red, orange, or yellow. This coloration may occur also in the specimen image and upset the color balance of the whole photomicrograph. It is therefore necessary not only to focus the image of the field diaphragm sharply but also to gauge this focus by consistently choosing the same color for the slight but inevitable color fringe at the edge of the blades. Some photographers halt adjustment at a blue fringe; others, at a green fringe. The aberration effects from these settings and many other factors can be accommodated by suitable filtering. The important thing to do is adopt one or the other color fringe all of the time in order to minimize and fix the aberration factor.

When the edge is blue or green in one sector but another color in an opposite sector, the lamp filament has not been aligned precisely enough. Steps 1 and 2 (page 28) should be repeated carefully. (See the bottom of the circle in Plate III-B.)

Even changing microscope slides may affect the focus of the condenser if the new slide has different thickness. Each time the slide is changed, the opening in the field diaphragm should be decreased and its image examined in the microscope.

Since the deleterious color effects of excessive chromatic aberration may be unpredictable, no suitable filter compensation can be applied. The only suitable compensation is readjustment of the condenser to the correct position. To adjust a condenser properly, move it up or down slowly until an image of the field diaphragm, as well as of the specimen image, is visible in the microscope. The field diaphragm image should be sharp and blue in color.

The problem of chromatic aberration is minimized when an achromatic condenser is used because it has correction for both chromatic and spherical aberrations. An achromatic condenser is therefore highly recommended for color photomicrography, particularly if apochromatic objectives are used. For best image quality, however, even an achromatic condenser should be focused critically.

Film-Emulsion Variance

The manufacture of color film involves the coating of light-sensitive emulsions, plus other materials, on a base. The thickness of each emulsion layer, measured in micrometers, must be carefully controlled. If the thickness of any one layer varies from its aim point, an effect on color balance will result. Color-balance variation permitted in manufacture is equivalent to the effect that a CC10 filter would produce on a film of normal balance. Experience has shown that this amount of variation is acceptable to most viewers. However, for critical use, CC Filters may be required to produce a desired color balance. A CC10 filter effect is the acceptable tolerance. The direction of variation could be in any one of the six colors: red, green, blue, cyan, yellow, or magenta.

When a photomicrographer changes from one film emulsion to another, the resulting photomicrograph may show an undesirable background color. The background should be white or very light gray. If it is not, this can be corrected in subsequent exposures by placing a CC10 Filter of a complementary color in the light beam. (See Table 9, page 46.)

When many rolls of a particular color film will be used over an extended period of time, several rolls of film with the same emulsion number can be purchased and stored in a refrigerator or freezer. If a deviation from normal color occurs in a film due to manufacturing difference, the amount of deviation can be determined by a filter-balance test with one roll. The test consists of making one exposure with no color-compensating filter and six exposures with a CC10R, CC10G, CC10B, CC10Y, CC10C, and CC10M filter, respectively. When all seven photomicrographs are placed on a suitable illuminator, one of them will probably show a clean white or very light gray background. In this way, the correct compensating filter can be determined.

Reciprocity Effect

Reversal color films of daylight type are normally designed for short exposure times—such as 1/30 second, 1/60 second, and 1/125 second. When the exposing illumination is correct, one of these short exposure times will usually produce normal color balance in the photomicrograph. Whenever the exposure time for a color reversal film is 1 second or longer, however, an undesirable color effect may be noticeable in the color photomicrograph. With certain color films, even an exposure of 1/8 or 1/4 second may produce a noticeable effect on color balance. If the light intensity is very low, a long exposure time is often necessary, resulting in a color shift. This color shift is due to the reciprocity effect. To explain: under normal conditions total exposure equals illuminance multiplied by exposure time ($E = IT$). *As the illumination level increases, exposure time decreases and vice versa,* in a reciprocal relationship. Throughout a normal range of light levels and exposure times, this relationship holds true, but in very low or extremely high light levels it may not. The photographic effect will vary with changes in illuminance (I) and time (T). This is especially true for long exposures, which are quite common in photomicrography.

Reciprocity effect is usually apparent as a decrease in emulsion speed at very low light levels. Since a color film contains three emulsion layers, a change in color balance occurs unless all three layers change alike. All three layers, however, may exhibit different reciprocity effects. Color balance can be quite erroneous when a very long exposure time is necessary. Long exposure times are often necessary in fluorescence photomicrography, as well as in photomicrography at high magnification, with polarized light, or with an interference microscope.

Color shift due to reciprocity effect can often be corrected by placing a color-compensating filter in the light beam before exposure. At very long exposures, two filters may even be necessary to compensate color variance.

Information on both exposure and filter compensation for reciprocity effect is given in Table 8. The data given in the table for each film applies only to the type of illumination for which the film is balanced.

Miscellaneous Factors

Several reasons for poor color balance are attributable to "improper handling" of color film, not to any specific photographic exposure technique or condition. In some cases, a permanent effect is produced, since no filter compensation can be applied. If a photomicrograph shows an undesirable color effect that is not due to any of the factors mentioned previously, one of the following conditions may be the cause.

Improper Storage: All photographic films are perishable products that are damaged by high temperature and high relative humidity. Color films are more seriously affected than black-and-white films because heat and moisture usually affect the three emulsion layers to different degrees. For color film, a change in color balance may be accompanied by a change in overall film speed and contrast. None of these effects is entirely predictable. Proper storage of color film is necessary both before and after exposure for consistent color balance. Of course, greater care is necessary under hot and humid conditions. Recommended storage conditions are covered in Kodak Pamphlet No. E-30, *Storage and Care of KODAK Color Films,* and in the Data Book *KODAK Color Films,* Kodak Publication No. E-77; available from Photo dealers.

Chemical Fog: Color films not sealed in foil envelopes or screw-cap cans should be kept away from any fumes, from formaldehyde, from paraformaldehyde, as well as from any other harmful gases or vapors. Such gases can influence color balance, speed, and contrast; their effects on color film are erratic and unpredictable. Color balance can vary in any direction. Film speed may increase, but contrast will usually decrease—especially with extended exposure to a particular gas, since maximum density is reduced.

TABLE 8

Reciprocity Characteristics of KODAK Color Films
(Exposure* and Filter Compensation)

Film	Exposure Time (Seconds)					
	1/1000	1/100	1/10	1	10	100
KODAK Photomicrography Color 2483	None	None	None	+ 1 stop CC20Y	+ 2½ stops CC40Y + CC10M	Not Recommended
KODACHROME II, Professional (Type A)	None No Filter	None No Filter	+ ½ stop No Filter	+ ½ stop No Filter	+ 1 stop CC10Y	Not Recommended
KODAK EKTACHROME 6116 Type B (Process E-3)	None No Filter	None No Filter	None No Filter	None No Filter	†	†
KODAK EKTACHROME 6115 Daylight Type (Process E-3)	None No Filter	None No Filter	+ ½ stop CC10B	+ 1 stop CC10B	+ 2 stops CC10B + CC10M	Not Recommended
KODAK High Speed EKTACHROME (Tungsten)	None No Filter	None No Filter	None No Filter	None No Filter	+ 1 stop No Filter	Not Recommended
KODAK High Speed EKTACHROME (Daylight)	None No Filter	None No Filter	None No Filter	+ 1 stop No Filter	+ 1½ stops No Filter	Not Recommended
KODAK EKTACOLOR Professional 6101, Type S	None No Filter	None No Filter	None No Filter	Not Recommended		
KODAK EKTACOLOR Professional 6102, Type L	Not Recommended			See Film Instructions for Speeds No Filter from 1/10 to 60 sec		
KODACOLOR II	None No Filter	None No Filter	None No Filter	+ ½ stop No Filter	+ 1 stop No Filter	+ 1½ stops No Filter
KODAK EKTACHROME-X	None No Filter	None No Filter	None No Filter	+ 1 stop No Filter	+ 2 stops CC15Y	Not Recommended
KODACHROME 25	††	None No Filter	None No Filter	††	††	††
KODACHROME 64	††	None No Filter	None No Filter	††	††	††

*The exposure increase, given in lens stops, includes the adjustment required by any filter or filters suggested.

†See Supplementary Data Sheet packaged with the film.

††At the time of publication, specific recommendations for reciprocity effects with KODACHROME 25 and KODACHROME 64 Films are not available. However, individual tests using 10 CC filter intervals are suggested. As a starting point, try red filtration for long exposures and cyan filtration for short exposures.

Note: The data for each film in the above table are based on average emulsions. They apply only to the type of illumination for which that film is balanced, and assume normal recommended processing. The data should be used as guides only. The adjustments are subject to change due to normal manufacturing variations or subsequent film storage conditions after the film leaves the factory.

Light Fog: Color films should be processed either in total darkness or in lighttight tanks. If a color film has received even a very short exposure to a darkroom light-leak or to a safelight, color balance will be affected. For example, a dark green safelight, which may be used briefly with panchromatic black-and-white films, will impart a green fog on color reversal films.

A loose camera back may allow some light to leak into the camera. The extent of "flare" in this case depends upon how long the condition exists.

Outdated Film: When possible, color film should be used before the expiration date stamped on the box because a change in color balance is possible after this date. The magnitude of change depends upon storage conditions during the usable "life" of that

TABLE 9

Color-Balance Corrections

Appearance of Photomicrograph	Possible Cause	Remedy
Slightly Yellow	1. Emulsion variance	Use CC10B filter.
	2. Colored mounting medium	Use blue CC Filter (CC10B or more).
	3. Low color temperature of light source	Use light-balancing filter of 82 Series.
Slightly Magenta (reddish blue)	Emulsion variance	Use CC10G filter.
Slightly Cyan (bluish green)	1. Emulsion variance	Use CC10R filter.
	2. Heat-absorbing filter in light beam	Use CC10R, or possibly CC20R, filter.
Slightly Blue	1. Emulsion variance	Use CC10Y filter.
	2. Abbé condenser not focused correctly	Adjust condenser for Kohler illumination.
Definitely Blue	1. Ultraviolet radiation present during exposure with arc lamp	Use No. 2B filter to remove ultraviolet radiation.
	2. High color temperature of light source	Use appropriate light-balancing filter of 81 Series.
Slightly Green	1. Emulsion variance	Use CC10M filter.
	2. Heat-absorbing filter in light beam	Use CC10M, or possibly CC20M, filter.
Slightly Red	1. Emulsion variance	Use CC10C filter.
	2. Abbé condenser not focused correctly	Adjust focus of condenser for Kohler illumination.
Slightly Yellow-Red	Low color temperature of light source	Use light-balancing filter of 82 series.
Definitely Reddish Yellow	Daylight film with tungsten source—no correction	Use No. 80A filter, plus light-balancing filter of 82 series.

film. If a film has been properly stored under recommended conditions, the rate of change is decreased but not eliminated. When a quantity of film is stored in sealed containers in a freezer at low temperature (0 to -10 F, for example), the rate of change is greatly decreased. In this case, film can be used beyond the expiration date without excessive change in characteristics. Film stored in this way should not be opened for use for at least 2 to 3 hours after removal from the freezer; otherwise, condensation might occur when the cold film is subjected to room temperature.

Radiation Exposure: Whenever a color film has received exposure from a source such as x-rays, radium, cobalt 60, or other radioactive materials, a change in color balance can occur. Such exposure can happen in hospitals, industrial plants, or research laboratories where either radiography or radiotherapy is practiced. If color films are stored or used in areas adjacent to a room where such radiation is present, suitable protection should be provided in the form of lead or concrete shielding of adequate thickness.

Processing Errors: Some changes in color balance in KODAK EKTACHROME Film and KODAK Photomicrography Color Film 2483 can be traced directly to a departure from recommended processing conditions. The color change can be toward magenta, green, green-yellow, or blue. The causes of such changes can be uneven or insufficient agitation, inadequate reversal exposure, one or more exhausted solutions, contamination of solutions, improper mixing of chemicals, or incorrect time or temperature. The resulting changes in color balance cannot be compensated adequately. The only remedy is correct processing, as recommended in instructions furnished with processing chemicals. Information on color variances due to processing is included in the following Kodak pamphlets: *Identifying E-3 Processing Errors* (E-60) and *Identifying E-4 Processing Errors* (E-62). Both are available on request.

Viewing Conditions: The conditions under which a transparency is viewed also may have a noticeable effect on its apparent color quality. For critical use, transparencies intended for projection should be judged by projection. If a transparency is to be viewed on an illuminator, such as in an exhibit or other display, the transparency should be judged on an illuminator similar to the one that will be used.

BLACK-AND-WHITE PHOTOMICROGRAPHY

Black-and-white photographic materials are often used in photomicrography for a variety of reasons. Illustrations in scientific books, journals, and reports are usually in black-and-white photography because of the additional expense involved in printing color. Although color might be more desirable, a good photomicrograph in black-and-white is always acceptable. (Figure 18.) Black-and-white materials have the advantages over color of better contrast control, more consistent development, and quicker results.

PROPERTIES OF PHOTOGRAPHIC MATERIALS

A black-and-white photomicrograph is almost always a *negative,* which must be printed onto a suitable positive material. Reversal materials are seldom used. The selection of a negative material, usually a film, requires knowledge of photographic characteristics such as color sensitivity, contrast, granularity, resolving power, speed, and exposure and development latitude.

Color sensitivity is an inherent characteristic relat-

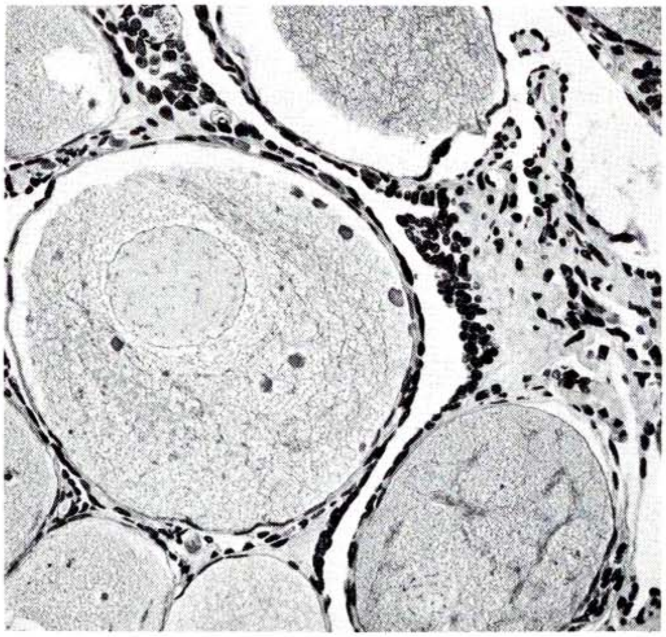


FIGURE 18—Same as cover, except photographed on KODAK EKTAPAN Film. A KODAK WRATTEN Filter No. 11 was used for correct tonal rendition of specimen colors.

ed to the response of a film to colors of the spectrum. A black-and-white film may be *blue-sensitive, orthochromatic, or panchromatic.* Blue-sensitive materials respond only to blue light, and to some extent to ultraviolet; orthochromatic materials have extended sensitivity in the green; and panchromatic materials are sensitive to all visible colors. Panchromatic films (or plates) are most often used in black-and-white photomicrography, since stained, colored specimens are common. Color sensitivity is a fixed property of photographic material; it is not subject to change by alteration in processing, as are other characteristics.

Generally, a film with very fine grain, high resolving power, and moderate contrast is selected for black-and-white photomicrography. Film speed is often a consideration, but is of lesser importance unless the subject is in motion and a film of high photographic speed is needed. In this case, a sacrifice in granularity, contrast, or resolving power may be necessary in order to record a satisfactory image.

The best procedure to follow in selecting a suitable material is to consider the specimen to be photographed. If the image exhibits relatively low contrast, a high-contrast film may be needed. Conversely, if the image has high contrast, a low- to medium-contrast film may give best rendition of detail. If the negative must be enlarged, a film having extremely fine grain can be an advantage.

Most films are classified as having low, medium, or high contrast. While this property can be altered to some extent by changing development time in a given developer or by changing the developer itself, the best practice is to follow the manufacturer's recommendation as to developer and development time. Deviation from an established procedure may have a pronounced effect on graininess, resolving power, or speed. An experienced photomicrographer, with a good photographic background, may be competent enough to vary development in order to produce a desired effect with a particular film, but the novice would do well to follow recommendations.

Resolving Power and Graininess

"Resolving power" refers to the ability of a photographic material to record fine details distinguishably. It is expressed as the number of lines per millimeter that are recognizable as separate lines in a photograph. Resolving power is determined for a particular film by photographing—at greatly reduced size—a parallel-line test chart with a high-quality lens. The image is then examined through a microscope to determine the number of lines that can be resolved. Determination of resolution depends on the test-object contrast; comparison of different films is made only with test-objects of equal contrast.

Graininess is related not only to the size of silver grains produced in a film after development but also to the irregular clumping of silver grains in the image. The degree of graininess will vary, depending on the type of developer used and, somewhat, on development time. A "fine-grain" developer may produce less graininess, but speed and contrast will be reduced. Generally, fast films have coarser grain than slow films.

The graininess designation for a photographic material is usually an indication of inherent resolving power. A film with very fine grain is thus *capable* of high resolution. Remember, however, that resolution in photomicrography is *not* dependent on the resolving power or on the graininess of a photographic material. High resolution can be achieved only by a quality microscope used efficiently. *Resolution in a microscope image cannot be improved photographically.* If the details in a specimen are not visible in the microscope, there is no film or technique that will make them visible.

It is a common *misconception* that a film with very fine grain is necessary to achieve high resolution in photomicrography. The real advantage of using a film with very fine grain is the probability of good image quality. Even though the recorded image cannot be better than the microscope image, the photographic material should not degrade image quality. A film with high resolving power will show an image more effectively than one of coarse grain or one with low resolving power. For example, a microscopic specimen contains fine details, whose separation is equivalent to about 2000 lines per millimeter. The microscope optics in use may resolve details at a magnification of 500. The separation of details in the image is now equivalent to 2000/500, or *only four lines per millimeter.* Any film is capable of such low resolving power, but a fine-grain film will show the details to better advantage than a coarse-grain one.

Selection of a fine-grain film will also allow some photographic enlargement without lowering image quality. Too much enlargement, however, may result in a state of empty magnification; that is, although the image is larger, no more details are resolved. This possibility must be considered when small-size films, such as 35 mm, are used in photomicrography. When an image is recorded on a small film area, further enlargement is necessary in order to distinguish details in a print. However, if maximum usable magnification has been achieved in the microscope and on the film, no further enlargement is possible without degrading image quality. When enlargement is contemplated, therefore, the magnification on the film should be lower than the maximum usable.

As an example, suppose that an image is recorded on film at ×500 but that the microscope optics in use are capable of ×1000. The recorded image could still be enlarged 2× without causing empty magnification. If the same image were recorded at only ×250, then a 4× photographic enlargement would be possible. If the image were recorded at ×1000, however, no more enlargement could be accomplished efficiently. These facts are of particular interest and importance when photomicrographs are made for publication.

Since the virtual image in the microscope is at 10 inches, the above comparisons refer to a glossy print viewed at 10 inches. Were a negative enlarged twice, the same detail would be observable at 20 inches, but no new detail would be opened up for study at 10 inches. Another factor governing the initial magnification is the N.A. of the system—a suitable objective for resolving the structures should be chosen and magnification at the film plane controlled with a good eyepiece.

Exposure Latitude

Exposure latitude is the range of exposure allowable from underexposure to overexposure, in producing a printable negative. More latitude is usually possible toward overexposure. When in doubt as to correct exposure, then, it is safer to overexpose a negative film (within reasonable limits). The amount of "acceptable" overexposure, however, is limited by increased graininess and difficulty in printing. Ideally, of course, the optimum exposure will produce the best negative.

Generally, high-contrast films provide the *least* exposure latitude, and low-contrast films the most. Also, latitude will vary, within limits, according to selection of developer and development time. As development time increases, exposure latitude as seen in the processed negative decreases.

Development Latitude

A negative film (or plate) capable of a wide range of contrasts (by manipulation of developer or development time) is highly desirable in photomicrography. Such a material can be used in a great number of applications. Very few materials, however, have this capability. A film designated as low in contrast can seldom be used to produce high contrast; one designated as high-contrast should be used cautiously for low-contrast results. In both cases, undesirable effects

TABLE 10

KODAK Sheet Films for Black-and-White Photomicrography

KODAK Film	Color Sensitivity	ASA Number (3200K)	Contrast	Resolving Power	Granularity
*EKTAPAN 4162	Pan	100	Medium	Medium	Very Fine
*PLUS-X Pan Professional 4147	Pan	125	Medium	High	Very Fine
*SUPER-XX Pan 4142	Pan	200	Medium	High	Fine
*TRI-X Pan Professional 4164	Pan	320	Medium	Medium	Very Fine
*ROYAL Pan 4141	Pan	400	Medium	Medium	Very Fine
*Contrast Process Pan Film 4155	Pan	80	Very High	High	Fine
*ROYAL-X Pan 4166	Pan	1250	Medium	Medium	Fine
*Contrast Process Ortho 4154	Ortho	50	Very High	Very High	Fine
*TRI-X Ortho 4163	Ortho	200	Medium	High	Very Fine
Commercial 6127	Blue Sensitive	8	Moderately High	High	Very Fine
*Commercial 4127	Blue Sensitive	8	Moderately High	High	Very Fine

*All of these films have ESTAR Thick Base.

on graininess, speed, and tonal rendition may result. A film of medium or moderate contrast will usually be capable of the widest development latitude.

Film Speed

Film speeds are arithmetic; the number assigned to a film as its ASA number is directly related to its sensitivity. A high-speed film of ASA 400 is twice as fast as one rated at 200, for example. Speed ratings are usually given on instruction sheets supplied with the different films or in Data Books, and apply for daylight or tungsten illumination as indicated.

The rated speed for a film, given in the instruction sheet, only applies when the film is developed according to recommendations. Deviation from the recommended development procedure may have a pronounced effect on speed and contrast.

SHEET FILMS AND PLATES

Although a variety of sheet films are available from Eastman Kodak Company, there are a few whose characteristics make them especially suitable for photomicrography. One, in particular, is KODAK EKTAPAN Film 4162 (ESTAR Thick Base). This panchromatic film has very fine grain, medium resolving power, and a speed of ASA 100. Contrast is moderate, but can be varied over a wide range from low to high by selection of developer and development time. EKTAPAN Film is capable of wide exposure latitude when developed for low to medium contrast. Its emulsion is coated on ESTAR Thick Base, which has excellent dimensional stability and lies flat. Because its speed is similar to that of KODAK EKTACOLOR Professional Film 6101, Type S, it is valuable when both color and black-and-white negatives are needed for the same subject. EKTAPAN Film is available from photographic dealers, in all standard sheet sizes.

Table 10 lists Kodak sheet films for black-and-white photomicrography and their pertinent characteristics. Development data for these films are included on instruction sheets contained in film boxes. These data can also be found in the Kodak Data Book *KODAK Professional Black-and-White Films* (Publication No. F-5), available from photo dealers.

There are two Kodak plate materials which are intended for use in photomicrography: KODAK M Plate and KODAK Metallographic Plate. KODAK M Plate is panchromatic, and is therefore useful for general photomicrography with colored specimens. KODAK Metallographic Plate, as the name implies, is designed especially for metallography (photomicrography of metals); it is orthochromatic in sensitivity. Both materials have medium grain and high resolving power. By developer selection, they are capable of a range of contrasts, from low to very high. Complete information about these plate materials is available from Eastman Kodak Company, Department 412-L, Rochester, New York 14650. Ask for *KODAK Metallographic and M Plates,* Kodak Publication No. P-33.

ROLL FILMS

The selection of a suitable roll film for general photomicrography is more limited than that of a sheet film. As in general photography, one of the most important characteristics to consider is graininess, since the recorded image must often be enlarged in order to distinguish fine details. Most roll films either have very fine or extremely fine grain. As previously stated, the condition of empty magnification must be considered when a roll film is used in photomicrography and a negative subsequently enlarged.

KODAK PANATOMIC-X Film is considered an excellent material for general photomicrography. It is available in standard 120 roll sizes and in 35 mm magazines of 20 and 36 exposures. PANATOMIC-X Film has extremely fine grain, panchromatic sensitivity, and moderate speed (ASA 32). An advantage peculiar to the 35 mm size is that it can be "reversed" to obtain direct positives by special development. Information about "reversal" of this film can be found in Kodak Publication No. F-19, *Black-and-White Transparencies with KODAK PANATOMIC-X Film (FX135).* For a copy, write Eastman Kodak Company, Department 412-L.

KODAK PLUS-X Pan Film is a 35 mm film available in magazines of 20 or 36 exposures. It has extremely fine grain, panchromatic sensitivity, and a speed of ASA 125. KODAK PLUS-X Pan Professional Film is available in packs and in 120 and 220 rolls. It has the same characteristics as the 35 mm film.

KODAK TRI-X Pan Film is a high-speed (ASA 400), panchromatic film with fine grain. It is especially suited to photomicrography of living, moving microorganisms, where short exposure times are necessary. It can be obtained in 120, 127, and 620 roll sizes as well as in 35 mm magazines of 20 and 36 exposures.

KODAK Photomicrography Monochrome Film (ESTAR-AH Base) SO-410 is a 35 mm high-contrast, extremely fine grain negative film. It features extremely high resolving power and moderate speed (exposure index 80 to 160, depending upon processing). This film is panchromatic, that is, it is sensitive to all colors. Photomicrographs of low-contrast specimens such as blood smears, tissue cultures, and karyotypes of human chromosomes will benefit from the contrast enhancement of this film. The contrast can be altered from high to medium by the development chosen. This film is available in 35 mm magazines of 36 exposures. Data on this film is available on request. See the inside back cover for details.

Complete film-development data and the characteristics of all standard Kodak black-and-white roll films are included in Kodak Data Book *KODAK Films for the Amateur,* available from photo dealers.

FILTERS IN BLACK-AND-WHITE PHOTOMICROGRAPHY

In black-and-white photomicrography, filters are used primarily for control of image contrast. An increase in contrast is often desired in order to make a specimen stand out against the background or to differentiate between colored elements, which may appear to have equal brightness on black-and-white film. In the latter case, a filter might be employed to absorb one color more than the other. Otherwise, the colors could record as equal gray tones, particularly if the panchromatic film used had equal sensitivity to both colors. (See Figure 19.)

When a specimen color is a very pale one against a bright background, it may record as a pale gray against a white background. A filter which absorbs this specimen color efficiently will render it as a darker gray; increased contrast will result. Maximum contrast occurs when the specimen color is completely absorbed; intermediate contrast, when it is partially absorbed; and minimum contrast, when the filter color is the same as, or similar to, the color in the specimen.

Increasing Contrast

Filters are often used to increase contrast in photomicrography of blood smears. In the United States, blood smears are commonly stained with Wright's Stain, a combination of methylene-blue and eosin stains. The first stain usually appears deep blue and the second light red. A green filter, such as a KODAK WRATTEN Filter, No. 58, will show more absorption for the eosin color and will render it with good contrast against the background. Another common stain combination, hematoxylin and eosin, usually requires a green filter also. The particular green filter depends on stain concentrations. The No. 58 filter applies if the eosin color is pale; a lighter green, such as a No. 11 or No. 13, when the color is more intense.

Occasionally, a specimen may be stained with more than two colors. In order to distinguish separate colors in a black-and-white photomicrograph, one must select a filter on the basis of its ability to adjust color brightness and to assist in recording the colors as suitable tones of gray. A KODAK WRATTEN Filter, No. 11, is recommended for this purpose when the film used is panchromatic. (See Figures 20 and 21.)

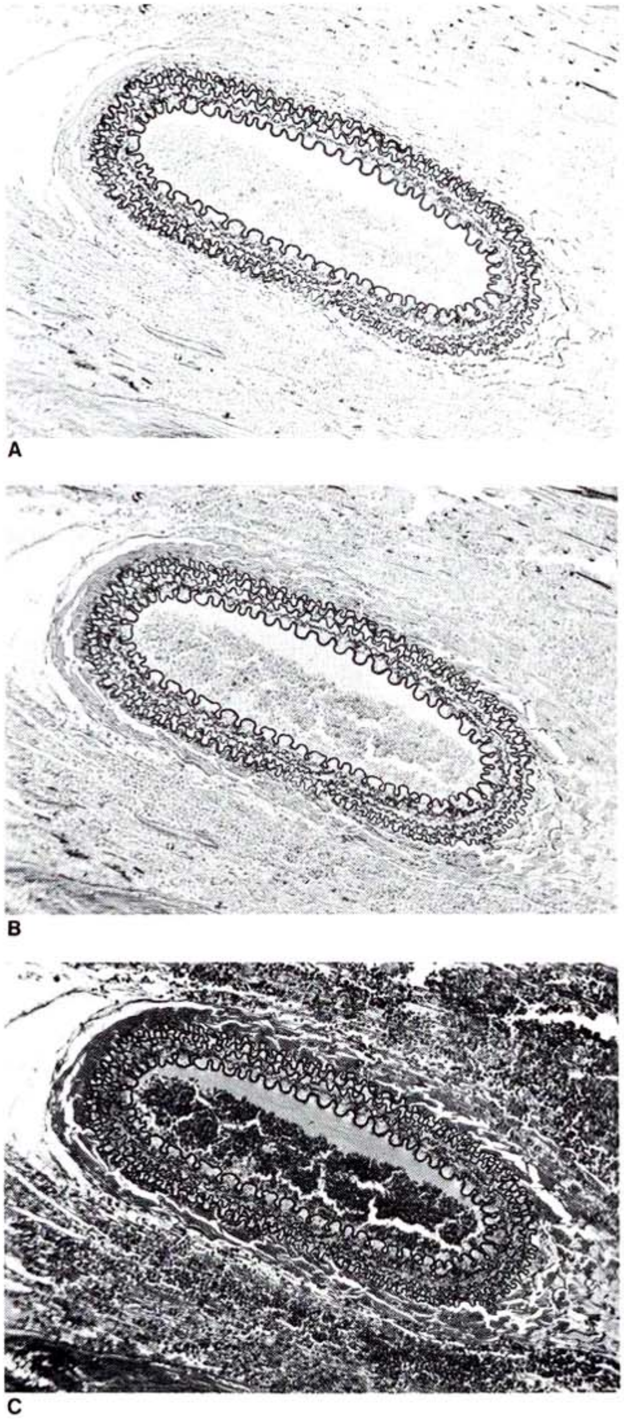


FIGURE 19—Effects of KODAK WRATTEN Filters in black-and-white photomicrography. A. No. 25 Filter (Red). B. No. 58 Filter (Green). C. No. 47B Filter (Blue).

When a specimen color is moderately dense, detail within the specimen can be recorded if the filter color is the same as, or similar to, the specimen color.

If maximum contrast is desired between a colored

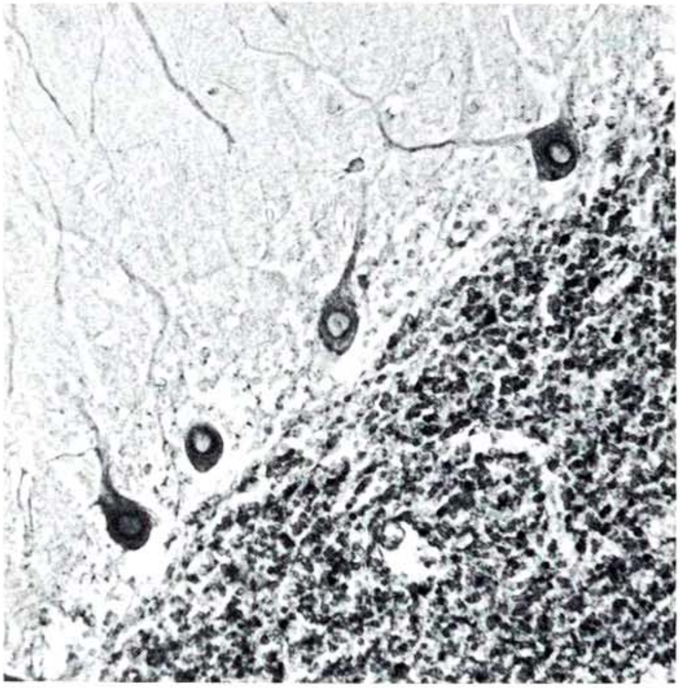


FIGURE 20—Same as Plate IV (page 40) on KODAK EKTAPAN Film. A KODAK WRATTEN Filter, No. 11 (light yellow-green), was used to lighten background and show detail in cells.

specimen and the background, the filter should absorb the specimen color completely. In this case, the filter color is *complementary* to the specimen color.

One technique which can be used for selecting a filter is to examine the specimen through the microscope while trying different filters in the light beam. When the desired contrast or detail rendition is achieved, a photomicrograph can be made through that filter on a panchromatic photographic material.

When contrast between specimen and background is desired, the following tabulation of specimen colors and contrast filter colors will be helpful as a general guide.

When selecting a filter for contrast, you must not obtain *too much* contrast in a photomicrograph. The result would be a blocking of dark areas and a loss of fine detail. Also, the use of high-contrast film is not necessary if sufficient visual contrast is obtained through the filter.

Specimen Color	Contrast Filter Color
Blue	Red
Blue-Green	Red
Green	Red
Red	Green
Yellow	Blue
Brown	Blue
Purple	Green
Magenta (Blue-Red)	Green
Violet	Yellow

KODAK WRATTEN Filters

Well over 100 different KODAK WRATTEN Filters are currently available as 50, 75, and 125 mm gelatin film squares. These gelatin film squares can be cut by the customer to fit specific equipment; they are available through photographic dealers.

Table 11 contains a list of the filters most often used in photomicrography. These filters can be combined for narrow-band transmittance or used individually to transmit the indicated broader bands of color.

TABLE 11

KODAK WRATTEN Filters for General Photomicraphy

Filter Number	Visual Color	Spectral Transmission
25	Red	From 590 nm into the infrared
58	Green	From 470 nm to 610 nm and 700 nm into the infrared
47	Blue	From 360 nm to 520 nm and 710 nm into the infrared
35	Purple	From 320 nm to 470 nm and from 650 nm into the infrared
22	Deep Orange	From 540 nm into the infrared
29	Deep Red	From 610 nm into the infrared
15	Deep Yellow	From 510 nm into the infrared
45	Blue Green	From 430 nm to 540 nm
11	Yellowish Green	From 300 nm to 350 nm and from 420 nm into the infrared

It is not always possible to recommend a specific filter, even when stain colors or other specimen colors are known. Visual inspection of a specimen through different filters is usually the best guide when selecting a filter for photomicrography. An experienced photomicrographer can examine a specimen and often select the best filter with little trouble, since he is usually familiar with stains and filters and with their effects in a photomicrograph.

The transmission and absorption characteristics of all Kodak filters are included in Kodak Publication No. B-3, *KODAK Filters for Scientific and Technical Uses*. This Data Book, published by Eastman Kodak Company, can be purchased through photo dealers.

PROCESSING THE NEGATIVE

After photomicrographic exposures have been made, the next step is to process the film. This includes developing, rinsing, fixing, washing, and drying.

Correct development is most important, since this part of the procedure causes the image details to appear on the film. For high negative quality, selection of the correct developer and developing time should be made according to the manufacturer's

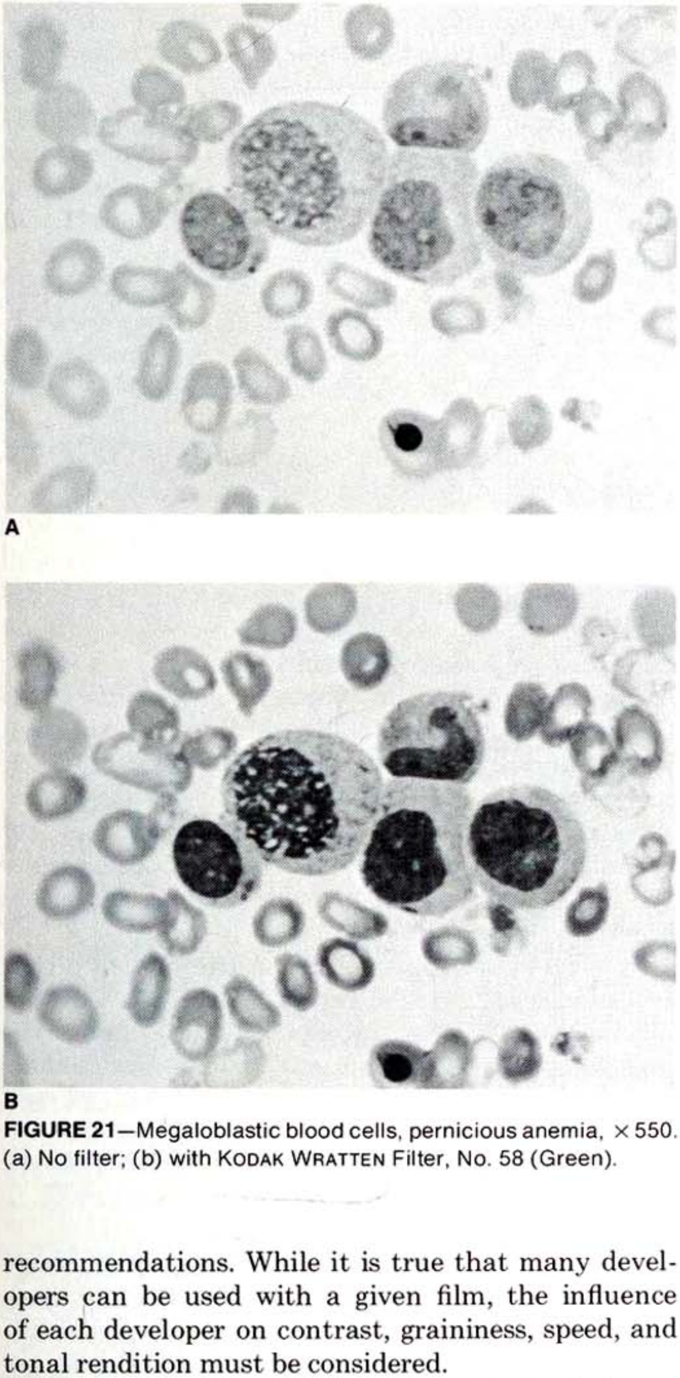


FIGURE 21—Megaloblastic blood cells, pernicious anemia, × 550. (a) No filter; (b) with KODAK WRATTEN Filter, No. 58 (Green).

recommendations. While it is true that many developers can be used with a given film, the influence of each developer on contrast, graininess, speed, and tonal rendition must be considered.

With continuous agitation, single sheets of film or single plates can be developed efficiently in a tray. When several exposed films or plates must be processed simultaneously, however, they should be developed in a tank with suitable hangers. Since intermittent agitation is used in tank development, developing time is usually about 25 percent longer than in tray development.

Development of a 35 mm roll film is commonly carried out in a reel-type tank, with agitation at 30-second intervals. The reel should be loaded in total darkness or with suitable safelight conditions, inserted into the tank containing developer, and the top set in place. Processing of the film can then be continued in room light.

After films or plates have been washed, it is recommended that they be immersed in KODAK PHOTO-FLO Solution for about 1 minute before drying. This helps to minimize the formation of water spots and uneven drying.

The following Kodak Data Books contain further information about handling and processing black-and-white films. They are available through photographic dealers: Publication No. F-5, *Kodak Professional Black-and-White Films,* Publication No. J-1, *Processing Chemicals and Formulas for Black-and-White Photography.*

PRINTING

Black-and-white photomicrographic negatives can be printed to a positive either on a suitable paper (producing a print) or on a positive film (producing a transparency). A negative can be printed by contact to produce a print the same size as the negative or by projection to produce a smaller print, one the same size as the negative, or a print on which the image is larger than the negative. For either contact or projection printing, a selection of Kodak photographic papers is available. Details concerning the characteristics of these papers are contained in Kodak Data Book G-1, *KODAK Photographic Papers,* available through photographic dealers. Two other Data Books, *Professional Printing in Black-and-White* (Kodak Publication No. G-5) and *Enlarging in Black-and-White and Color* (Kodak Publication No. AG-16), will also be of value.

For paper prints, it is common practice in photomicrography to use glossy-surface papers. Glossy paper will produce prints with the highest brilliance and best rendition of details. Glossy prints are also commonly required for reproduction in journals, books, and other publications.

If a photomicrographer already has negatives, but needs positive transparencies, he can use one of two Kodak films to obtain transparencies: EASTMAN Fine Grain Release Positive Film 5302 or KODAK Fine Grain Positive Film 7302. The first is a 35 mm film available in 100-foot rolls only; the second is available in sheets and rolls. Both films are blue sensitive and of low speed. They can be handled and processed under the illumination from a KODAK Safelight Filter, No. 1A (light red), in a suitable lamp.

For a complete guide to all Kodak black-and-white materials, processing, printing, enlarging, and copying, all in one publication, we recommend the *KODAK Darkroom DATAGUIDE,* available through your photographic dealer.

EXPOSURE METHODS

As in other forms of photography, exposure in photomicrography is the result of light acting upon a sensitized emulsion—the film. Exposure is influenced by light intensity (image brightness) and by exposure time. In order to record an image of brightness, a specific exposure time is necessary, depending upon the film speed.

Exposure time can be determined either by using a sensitive exposure meter or by making an exposure test series. Because image brightness in photomicrography can be quite low, particularly at high magnification, a preferable exposure meter should be sensitive to very low light levels. To determine exposures with a hand-held meter, hold the meter above the eyepiece at a distance such that the diameter of the emerging light-beam will be slightly greater than the diameter of the photocell. A meter reading is made with the meter in this position in a dimly-lighted room. Some professional exposure meters have special microscope attachments. The manufacturer's manual gives instruction for these attachments.

When an exposure test series is to be made, any necessary filters should be placed in the light beam, whether a color film or a black-and-white film is to be exposed.

EXPOSURE TESTS

Roll Film

When a 35 mm film or other size of roll film is to be exposed, an exposure test can include several frames, each exposed for a different length of time. Image brightness, of course, should remain constant. The exposed film is then processed and the individual frames are examined over an illuminator to determine which one received the correct exposure.

The most convenient series would include all of the exposure times available with the camera shutter. These can range from 1 second to 1/125 second. If the shutter has a *Time* (T) setting, a few long exposures could also be made (2, 4, and 8 seconds, for example). One out of this wide range of exposure times will be correct for that film and the existing image brightness.

Another convenient method of determining proper exposure is to make an exposure series on a roll of black-and-white film, process it, and evaluate the negatives. The best negative is the one that exhibits the most detail throughout, in both shadows and

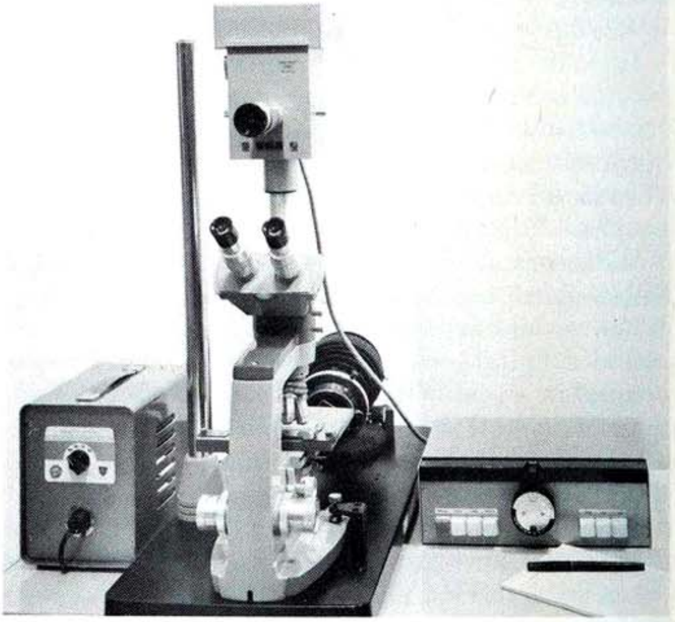


FIGURE 21—Photomicrographic setup with automatic exposure meter attachment.

highlight. The film speed of the black-and-white film can then be correlated with any other film that might be used. As an example, suppose the taking film was Kodak Panatomic-X Film with a speed of ASA 32 and the new film to be used is Kodak High Speed Ektachrome Film (Tungsten) with a speed of ASA 125. The proper exposure on the Panatomic-X Film turned out to be 1/30 second. Since High Speed Ektachrome Film has about *four times* the speed of Panatomic-X Film, it will require only *one-fourth* the exposure, or 1/125 second. (Exposure time varies inversely as film speed rises.)

Sheet Film

When a camera accepts sheet films, a series of test exposures can be made on one film sheet by the following procedure:

1. Pull out the dark slide of the film holder until the entire sheet is uncovered in the camera. Make an exposure for 1 unit of time; for example, 1 second, 1/2 second, 1/125 second, or whatever initial time may be indicated by the image brightness level.
2. Push in the slide about an inch and *repeat* the exposure for the same unit of time.
3. Push in the slide another inch and give an exposure for 2 units of time.
4. Continue to push in the slide to cover approximately 1-inch steps, exposing each step for twice as long as the previous one. The successive steps will then have received exposures of 1, 2, 4, 8 times the initial time unit.

Develop, fix, and wash the film as recommended in the instruction sheet. Examine the resulting negative to select the step which shows the best reproduction of the subject.

EXPOSURE CALCULATION

Once the correct exposure time has been found for a particular set of optical conditions, the correct times for new conditions *with the same microscope and illumination* can be calculated. The factors which affect image brightness are changes in magnification or in numerical aperture. Magnification, of course, will change if a different eyepiece, a different objective, or a new eyepiece-to-film distance is involved. Also, if the objective is changed to one of higher or lower magnifying power, the numerical aperture will be different.

If the optical conditions for which the exposure time was originally determined by test are called "standard," they can be compared mathematically with the "new" conditions to calculate the "new" exposure time, according to the following formula:

$$\frac{\textit{New Exposure Time}}{\textit{Standard Time}} = \left(\frac{\textit{Standard N.A.}}{\textit{New N.A.}}\right)^2 \times \left(\frac{\textit{New Magnification}}{\textit{Standard Magnification}}\right)^2$$

The application of this formula is illustrated by the following example: The correct exposure for Kodak High Speed Ektachrome Film was determined to be 1/100 second (0.01 second). Suppose that a 10× eyepiece and a 20× objective (N.A. = 0.50) were used. Magnification is ×200. These are the "standard" conditions: exposure is 0.01 second, magnification is ×200, and N.A. = 0.50. The same film will be exposed, of course, but the new exposure will be made at ×430, with the same 10× eyepiece and a 43× objective of N. A. = 0.65. What is the new exposure time under these conditions?

$$\frac{\textit{New Exposure}}{.01} = \left(\frac{0.50}{0.65}\right)^2 \times \left(\frac{430}{200}\right)^2$$

$$\textit{New Exposure} = .0276 \textit{ second} = \frac{1}{30} \textit{ second}$$

In this way exposure time can be calculated when any of the optical exposure factors are changed. It is even possible to set up a table to include all exposure times for a color film used under different conditions. This would assume, of course, that the microscope and the illumination were correctly adjusted for each condition.

In black-and-white photomicrography, specific light filters are often used for contrast control. When different filters are used, as they often are, their "filter factors" must be considered in working out exposure determinations.

Judgment of Exposure

A good photomicrographic exposure should always show the subject to best advantage, whether the image is on a reversal color film or on a negative material. It is easier, however, to judge correct exposure on a reversal material, since this type of film has very narrow exposure "latitude." If an exposure time is not right, this is immediately apparent. Overexposure will wash out the light areas, with a loss of "highlight" detail. Underexposure will cause a darkened appearance overall, with loss of detail in the darkest, or "shadow," areas. A good exposure on a reversal film will show detail in all areas where detail was evident in the specimen.

Negative materials, both color and black-and-white, exhibit much more exposure latitude than do reversal color film. Therefore, judgment of the best exposure time is more difficult. In general, exposure should be controlled to provide best rendition of detail in the *darker* areas of the subject. These areas will be represented on a negative by light areas. If the film is underexposed, very little detail will be evident in these areas of the negative. When such a negative is printed, the background and highlight areas may print very well, but the darker areas will be too dark, with few, if any, details. Considerable overexposure of a negative may cause loss of detail in both the highlight areas and the darkest areas. With negative materials, more overexposure than underexposure can be tolerated.

Filter Factors

When a colored filter is placed in the illumination beam in black-and-white photomicrography, it will *absorb* a certain amount of light. The amount of light absorbed depends upon the particular filter. One may absorb more, or less, than another. If the exposure time for a black-and-white film is determined with "white" light—that is, without any filter—it must be adjusted (increased) when a filter is used, since the image brightness will be *decreased.* The amount of increase in exposure time is known as the "filter factor." The exposure time without the filter is multiplied by the filter factor to determine the exposure with the filter in place. Filter factors for all Kodak black-and-white films are published in the instruction sheets packaged with the films.

If exposure time is determined for a black-and-white film by making exposure tests, the filter can be in position. Correct exposure will then be achieved without resort to the filter factor. If a new exposure of a different subject is to be made, and a different filter is necessary, the new exposure *must* consider the change in filter factors. For example, suppose that an exposure was made on a black-and-white film, with a filter having a factor of 4. The new exposure requires a different filter, whose factor is only 2. The new exposure time will be *one-half* the previous one. Or, if the new factor were 8, the new exposure time would be *twice* the original exposure.

If exposure is determined with an exposure meter, only white light (unfiltered) should be used for black-and-white films. The photocell in an exposure meter does not have a uniform response to all spectral colors, so measurement of exposure with the filter in place could be erroneous. Thus, the best practice is to measure the exposure time for white light and then apply the filter factor for finding the correct exposure.

If color films are to be exposed, filter factors do not apply. Light-balancing filters and color-compensating filters are used here. They are already in place for any exposure determinations.

Determining Filter Factors: Because of variance in the spectral response of different photographic materials and the difference in color quality of different types of illumination, published filter factors can only be considered as approximate. If an exact filter factor is necessary for specific conditions, it must be determined experimentally. This can be accomplished by making a "step-exposure" series on the film to be used, with the illumination to be used, and of course with "white light." It is not necessary to have a specimen in place on the microscope stage for the exposure series—the background brightness will suffice. The exact series of exposures will be governed by the actual brightness level. If the level is low, a "power of 2" series—such as 1/2, 1, 2, 4, 8, and 16 seconds—could be used. Shorter exposure times would apply for a high brightness level. Microscope adjustments must not be changed during the test.

If a corresponding step-exposure series were then made with a filter in place, both series could be compared to determine which steps matched for density. If no two steps matched, either another series with closer steps could be made or the filter factor could be determined by interpolation of density between the two steps most closely matched. This requires a knowledge of densitometry, but is the most exact technique.

Suppose, for example, that 2 seconds of exposure produced a medium density in a series made with white light. When the filter was placed in the light beam and another exposure series made, it took 8 seconds to produce a density equal to the 2-second exposure in the first series. The filter factor would then be 8 divided by 2—which, of course, is 4.

Experimental determination of filter factor is especially useful when filters are used in combination, since factors are seldom published for combinations. Once the combination filter factor has been determined, it can then be applied in all future exposures, provided that the same film and illumination are used.

Exposure Meters

Some types of exposure meters are made specifically for photomicrography and are sensitive enough to respond to light through a wide range, from very low to very high brightness. A meter scale may be precalibrated by the manufacturer to give a direct reading of exposure time, with various settings for film speed. Obviously this is the most convenient type of meter. Other meters may be very sensitive to light but have readings in terms of units of illuminance, such as footcandles. In this case, the manufacturer often provides a simple calculator, table, or graph so that brightness readings can be converted to exposure times.

If a meter reads brightness but no device is provided for correlating brightness and exposure time, the meter must be calibrated. This can be done by making a brightness reading of the image at low magnification (about ×100). The brightness reading is recorded, as indicated on the meter scale, for later reference. Then a series of exposures is made at various shutter speeds on a reversal color film. After the film is processed, the correct exposure time is selected. A table (or a graph) can then be made that includes the brightness reading, the correct exposure time for that reading, and the speed of the color film exposed. If another reading were made at a different magnification, one could use the existing data to find the correct exposure time. For example, if the new reading at lower power indicates *twice* the brightness, then the new exposure time will be *one-half* the previous one; if a new reading at higher power is only one-half the first one, the new exposure time will be *twice* as much, etc. Exposure time for a given film will vary *inversely* as the brightness of the image increases. Table 12 is shown as an example. The figures are arbitrary and do not refer to any particular conditions. Film speeds are included to make the table complete, but they do not refer to particular films.

TABLE 12

Brightness Readings	Shutter Speeds			
	ASA 32	ASA 64	ASA 125	ASA 250
500	1/125	1/250	1/500	1/1000
250	1/60	1/125	1/250	1/500
125*	1/30	1/60	1/125*	1/250
64	1/15	1/30	1/60	1/125
32	1/8	1/15	1/30	1/60
16	1/4	1/8	1/15	1/30

*Exposure time determined by test for a brightness reading of 125 (example only).

This system could be used for any light meter not calibrated in terms of exposure time or film speed, including meters normally intended for conventional photography outdoors and indoors.

"Probe" Meter: One type of meter is equipped with a "probe" containing a photocell. The probe is placed in the microscope eyepiece tube, with the eyepiece removed, in order to read image brightness. When a probe type of meter is used, the *same* eyepiece should always be used in the microscope for photography. Otherwise, the meter readings will not always be valid.

When an eyepiece-camera with a beam splitter is used over the microscope, light readings can be made from the light emitted from the eyepiece of the beam splitter. However, image brightness here will often be *less* than that "seen" by the film because the division of light varies with different beam splitters. In some systems, 90 percent of the light goes to the camera and only 10 percent is seen visually through the observation eyepiece. The actual division, however, may be 80-20, 70-30, or even 50-50, depending on the specific eyepiece camera in use. Hence, the amount of light division will influence the calibration of a light meter.

Making Exposure Readings

Exposure readings can be made in various positions: at the film plane of the camera, anywhere between the eyepoint of the ocular and the film plane, in the eyepiece tube of the microscope with the eyepiece removed, or from the light emitted from the observation eyepiece of a beam splitter. The best position, of course, is at the film plane, since the image brightness there is the same as would be recorded on the film. This position, however, is not always accessible because the camera may be closed during photography. The ground-glass screen of a sheet-film camera allows this type of reading very nicely.

If the exposure reading is made at some position above the microscope eyepiece, the image brightness will always be *greater* than that at the film plane. This fact must be considered in calibrating an exposure meter. Also, the light reading should always be made at the same distance above the eyepoint.

Methods of Measurement: Wherever the reading is made, there are two methods of measuring brightness, or exposure time. One method is to read just the background brightness with the specimen slide moved aside on the stage. This system provides a large area of uniform brightness on which to make a reading. The method is especially useful with reversal color film, where exposure time is dependent on the brightest part of the specimen—which is essentially the same as the background brightness.

The second method of measuring brightness is to read the actual brightness of the specimen image. This system also works efficiently for color films, as long as the specimen image has average brightness. If dense areas predominate the field, overexposure may occasionally be encountered, and the bright, highlight areas may appear washed out. This method should always be used, however, when negative films, either color or black-and-white, are to be exposed. This produces good rendition of detail in the darker areas of the subject as previously described in "Judgment of Exposure."

Whichever method is used to make the reading, the meter must be calibrated to conform with it, if accurate readings are to be made consistently.

Integrating Meters: Many current photomicroscopes designed for brightfield photomicrography, and others involving phase and interference systems, are equipped with photocells or photoresistors that integrate and measure the brightness of the image. This is integrated with other control data, like film speed, to automatically time the exposure. A wide range of brightness is accommodated. Allowances for filter factors are made by the integrating element. But ASA ratings, reciprocity departures, and subject density can be preset by the photographer. Those who practice photomicrography extensively would do well to study the various types of these meters offered by manufacturers.

EXPOSURE RECORD

When photomicrographs are made often, it is advisable to make a record of the conditions involved. A detailed record will permit duplication of a setup for future use either in rephotographing a particular specimen or for photography of similar specimens. This information could be recorded on the envelope containing a photomicrograph, or in a notebook. The following table includes pertinent data for one particular situation, as an example.

Photomicrograph No. 69-104

Subject	Pollen grains—hibiscus
Stain	None, normal color
Objective	10× apochromat
Eyepiece	15× compensating
Distance (Eyepiece to Film)	10 inches (fixed)
Magnification	×150
Light Source	Tungsten halogen, 12 volts
Film	KODAK EKTACHROME Film 6116, Type B
Filter	CC10R
Exposure Time	1/25 second
Development	Process E-3
Remarks	Above filter used to neutralize color of heat filter.

The data may differ for some photomicrographs. Exposures for black-and-white films, for example, should include specific developer, time, and temperature. If the eyepiece-to-film distance is always fixed, as in most eyepiece-cameras, this datum can be omitted. The purpose of the photomicrograph could also be included (for whom it was made, why it was made, etc).

COMMON FAULTS IN PHOTOMICROGRAPHY

Even the most careful photomicrographer sometimes makes mistakes and produces a photomicrograph of less than the best quality. In order to correct such mistakes in future work, he must be aware of causes and effects. There are many factors which can affect resultant image quality. Some are related to low-quality optics, while others are related to improper adjustment and alignment of the illumination and optics. The specimen itself can influence image quality; it may be too thick or be improperly stained. Dirt, dust, or grease on any of the optical components can affect the resultant image. Incorrect use of filters in black-and-white photomicrography can reduce contrast or detail rendition. In color work, the wrong filters will affect color balance.

MORE-COMMON FAULTS

Unsharp Image

This is probably one of the most common undesirable effects in photomicrography. There are several causes:

Possibly the image was focused sharply but there was some "slippage" in the fine-focus adjustment on the microscope. If this happens often, the microscope may be too old or worn or the adjustment may be loose. You can find out whether the image focus changes by watching it for a short while after critical focus. If it stays in focus, something else is causing the trouble.

The camera shutter may have been actuated too hard or too fast—causing vibration which made the image go out of focus. Always use a cable release and press it slowly.

If the image is focused on a ground-glass screen, it may be that the surface is too coarse, making critical focus difficult. Use a ground glass with a clear center so that focus is achieved on the "aerial" image. This situation was discussed under "The Ground Glass" on page 20.

Most eyepiece-cameras include a reticle in the observation eyepiece. This reticle must be sharply focused. A young person's eyes will accommodate the focus of this reticle even when it is not in sharp focus.

Check before making the exposure to be sure that both the reticle and the specimen are in focus. If the reticle is out of focus, then the specimen image may also be unsharp when it is recorded on film. A focus adjustment for the reticle is provided on the observation eyepiece.

If a high, dry objective is used in the microscope, and if the cover glass on the specimen is too thin, undercorrection for spherical aberration is introduced. The image can never be focused sharply. There are only two remedies. (1) If the cover glass can be removed and replaced with one of correct thickness (No. 1 1/2), a better image will be obtained. This remedy, however, is not always possible, since the cover glass may be cemented firmly in place. (2) An oil-immersion objective of comparable magnifying power may be used to obtain a better image. Then the cover-glass thickness is less important. Objectives of this type are available from several firms. If one is to be purchased, make sure it can be used efficiently with the microscope in use.

Possibly the objective in use was designed for a different microscope with a different tube-length. This may also introduce spherical aberration, affecting image sharpness.

People who wear glasses or contact lenses sometimes have difficulty achieving sharp focus in a microscope or through an observation eyepiece on a beam splitter. The only thing that can be done here is to check with the manufacturer. Possibly he is aware of this problem and has a means for solving it. Sometimes a high eyepoint eyepiece can be used effectively to correct this problem.

Vibration will cause a recorded image to be unsharp, particularly at high magnification or with long exposure times. If any steady bench vibration occurs, it will be noticeable in the microscope at high magnification; the image will appear to be in motion. A fast shutter speed (1/100 second or faster) or electronic flash will minimize the effect, but will not remove the cause. If the reason for the bench vibration can be found, possibly it can be eliminated. The alternative is to mount the microscope or the stand on suitable shock absorbers. Shutter vibration, too, must be suspected. A slow shutter speed will minimize this effect because if the cause arises in the shutter, this vibration is only prevalent during the first part of the exposure.

Hazy Image

An image appears hazy when a grease spot is present on the front lens of the objective, on the top lens of the eyepiece, or on the specimen slide. These surfaces may have been touched accidently. All glass surfaces should be inspected frequently and cleaned when necessary. A piece of lens tissue can be moistened with xylene (EASTMAN Organic Chemical No. P460) or with the more volatile ligroine (EASTMAN Organic Chemical No. P950) to clean a greasy surface. Too much solvent, however, can affect the cement in the objective lens or the mounting medium on the slide.

A small drop of immersion oil on a dry objective will also produce a hazy, unsharp image. This is usually an accident and can occur when a dry objective is placed in position with oil on the slide. The oil can be removed with tissue moistened with xylene. Oil should always be removed from a slide after use.

Uneven Illumination

When the objective and substage condenser are not aligned satisfactorily, or when the illuminator and the light source within it are not correctly aligned with respect to the microscope, the illumination will be uneven. The effect will be quite noticeable in a recorded image. The background, or the specimen image, will be darker on one side than the other.

The objective in a microscope is usually fixed in position, so no adjustment of its position is possible. The substage condenser, however, can and should have centering screws so that it can be centered with respect to the objective. To find out if it is off-center, look down the tube of the microscope with the eyepiece removed. A circle of light will be seen which is the back lens of the objective. Then, if the substage diaphragm opening is decreased, the circular image of the diaphragm imposed on the image of the back lens will be seen. If the two images are concentric, then the condenser is aligned with the objective. If they are not concentric, adjust the screws on the condenser until they are.

When the objective and condenser are properly centered with respect to each other, but the illumination is still uneven, either the illuminator is off-center or the lamp in the illuminator is off-center. Very often the lamp itself is off-center, particularly with built-in illumination. The position of the filament may vary in different tungsten lamps, even in those of the same type. Most microscopes with built-in illumination have centering screws on the lamphouse for centering the lamp. This centering can be checked by placing a piece of white paper in front of the field lens and adjusting the centering screws. When the lamp filament is off-center, the illumination on the paper will be uneven. Adjust the screws until the light is even. This effect may also be seen by looking in the microscope *without* a specimen in place—just the clear field will be visible. If the light is too intense, place a neutral density filter in the light beam to reduce the light level. The effect of uneven illumination, whatever the cause, is most noticeable at low magnification, since a large field of view is recorded.

Low Contrast

There are three principal reasons for low contrast in a photomicrograph. First, the substage diaphragm may be opened too far, creating flare and reducing image contrast considerably. The setting of this diaphragm should be made according to the principles of Kohler illumination. (See page 28.) The effect is noticeable in both color and black-and-white photomicrography.

Second, a filter may sometimes be used in black-and-white work for detail rendition with stained specimens resulting in low contrast. If the color of the filter is similar to that of the specimen—it transmits the color of the specimen. The effect is to reduce visual contrast between the specimen and the background. Contrast in this case can be enhanced by (1) using a filter which has partial absorption for the specimen color, (2) using a film of higher than normal contrast, or (3) selecting a developer that produces higher contrast.

Third, the subject itself may exhibit very little contrast. This contrast can be improved to some degree by the previous mentioned methods. Different lighting methods may improve this contrast also.

LESS-COMMON FAULTS

Too Much Contrast

Too much contrast is not likely to happen with color film, since processing conditions are fixed and contrast filters do not apply. It occurs in black-and-white photomicrography (1) when a high-contrast film is used and no effort is made to select a low-activity developer, (2) when a high-contrast developer is used with a regular film, (3) when development time is unduly prolonged, or (4) when a contrast filter is used erroneously.

Poor Resolution

Probably the principal cause of poor resolution is improper use of the substage diaphragm—when it is reduced to too small an opening. Resolution is decreased considerably, artifacts and diffraction are introduced, and a generally poor image results. Follow the technique of Kohler illumination in adjusting the diaphragm to the correct opening.

If the substage condenser is not correctly adjusted and its position is too low, the effects are similar. The objective is not used at full numerical aperture.

Poor resolution also occurs when a photomicrograph is enlarged too much. Empty magnification results, and the image appears unsharp. When selecting the magnification, follow the rule of 1000 times the N.A. of the objective. At times, overmagnification may be permissible when the purpose is to make fine detail larger and therefore, easier to see—a psychological benefit.

Bright Spot in Field

A bright spot in the field can occur when one uses a conventional camera with an integral lens or an eyepiece-camera with a compensating lens above the microscope eyepiece. Ideally, the eyepoint of the ocular should be at, or very near, the front surface of the lens. If the front surface of the lens is too close to the ocular, an out-of-focus image of the back lens of the objective may be recorded as a bright spot, when a reversal color film is used. It would, of course, be a *dark* spot on a negative, becoming a bright spot in the print. If the camera can be moved up a little farther above the microscope, the spot will disappear. If it is moved too far, however, image quality may suffer, or the image may be vignetted.

Shutter-Blade Image

If the camera shutter is too far above the eyepoint, a silhouette image of the shutter blades may be recorded as they open, particularly with fast shutter speeds. The shutter (leaf type) opens from the center outward, then closes toward the center. If the eyepoint of the ocular is positioned near the center, this effect cannot occur. Of course, if a lens is in the camera one has little control of the eyepoint position, other than to place it at or near the front surface of the lens. In this case, fast shutter speeds (1/60 second or shorter) should not be used. If necessary, a neutral filter can be used to reduce the light, thus permitting slower shutter speeds. When the camera does not contain a lens, the eyepoint can be positioned in the center of the shutter.

Out-of-Focus Spots

Dust and dirt particles on the front surface of the lamp condenser lens, on filters in the light beam, or on the top surface of the eyepiece will record as out-of-focus spots. They may be colored on a color film or gray (or black) on a black-and-white film.

Scratches on a glass surface will record as unsharp spots. Bubbles in a lamp condenser or in a heat-absorbing filter in the illuminator will show as spots also, and may be bluish in a color film record. Cracks in those elements will produce streaks. Check all glass surfaces frequently, especially if unidentified spots show up.

SPECIALIZED TECHNIQUES AND APPLICATIONS

SPECIALIZED TECHNIQUES

A majority of the subjects examined or photographed through a conventional microscope appear either dark or colored against a light background. When they appear dark, it is due to light absorption within their elements. When they appear colored, it is usually because they have been treated with biological stains to produce color contrast. This color contrast is a result of differential absorption of various stains by the elements of the specimen. Whether the subject appears dark or colored, the effect is called "brightfield illumination."

In an unstained condition, many specimens exhibit little or no contrast when viewed against a bright background in a compound microscope. They are colorless and comparatively transparent. Consequently, they are practically invisible. When staining a specimen is either impossible or undesirable, conventional brightfield microscopy cannot be used. Another microscopical technique must be used to make the specimen visible. The particular method selected depends upon the specimen itself and the particular results desired. The following discussions are intended to present brief descriptions of those special techniques that will either produce better optical contrast between the elements of a specimen and its background or allow images to be viewed or photographed with finer resolution.

Contrast Systems

Several simple and sophisticated optical arrangements have been worked out for the purpose of enhancing the contrast between specimens and background and for delineating structures in transparent and translucent subjects. The photomicrographer does not need a doctorate in optics to use such microscopes. Manufacturers' instructions are explicit, operationally. However, their technical discussions do require some knowledge of the physics of light for understanding. The designs involve numerous slits, plates, prisms, and lens and condenser combinations. The photomicrographer can best juggle these, and most confidently evaluate his results, when he has a grasp of the underlying principles. When he does not know them, he is in the position of someone driving a car without knowing of the existence and function of the transmission.

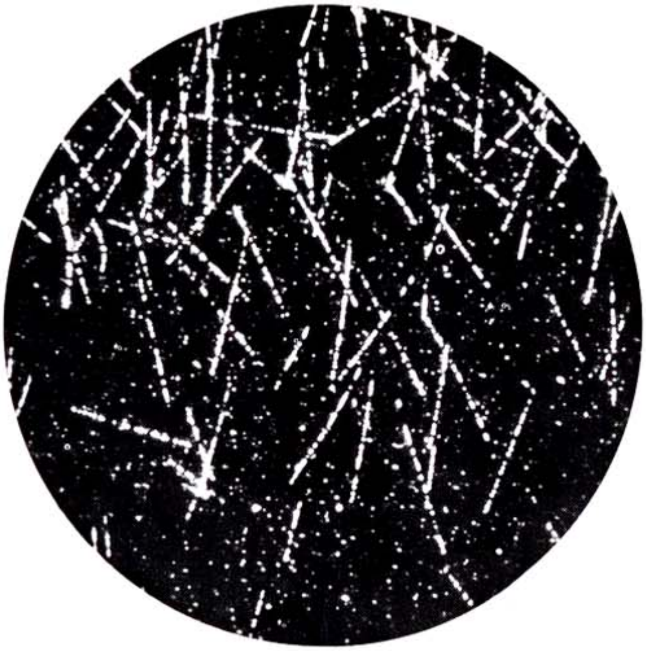


FIGURE 23—Alpha particle tracks, × 500, with darkfield illumination. Photographed on a KODAK Metallographic Plate.

There is another benefit that comes from being knowledgeable. Many of the devices are quite intricate. Tolerances of the order of a wavelength of light are involved. Therefore, educated prudence demands that the photomicrographer confine himself to making only the operating adjustments and alignments. Any other manipulations or repairs should be left to the manufacturer or his representatives. The photographer should know what to leave alone.

Darkfield Method

Many transparent and semitransparent specimens—such as microorganisms, cell structures, and crystal inclusions—are not readily visible in a brightfield microscope. Their visibility can be improved greatly by a method called "darkfield illumination," in which the specimen is seen as a bright object against a dark, or even black, background.

For the darkfield method, the cone of light normally illuminating the specimen must not enter the microscope objective. In the darkfield microscope, only light that is scattered or reflected by the specimen is "seen" by the objective. This is achieved in the conventional microscope by use of darkfield diaphragm stops or special darkfield substage condensers called "paraboloid" or "cardioid" condensers. The darkfield stop is inserted underneath the condenser and the aperture diaphragm is opened to its maximum. It may be used at low and medium power with dry objectives, while the special condensers are used only at high power. The N.A. of this condenser is greater than the N.A. of the objective. The cardioid and paraboloid condensers use the oil-immersion technique, which requires that oil be placed between the bottom of the glass slide and the top of the condenser. Great care must be taken that bubbles are not introduced either in the oil under the oil-immersion objective or in the oil between the slide and condenser. A single bubble will introduce considerable flare and reduce both image contrast and optical quality.

Since special darkfield condensers are to be used with oil, particular care must be taken in regard to slide thickness. The correct range of slide thickness is usually specified on the condenser mount. If the slide is too thin, the oil layer collapses when the condenser is focused critically. If the slide is too thick, it is often impossible to obtain correct focus of the condenser.

The essential principle of the darkfield optics is the formation of a hollow cone of light whose apex occurs in the plane of the specimen. When the light is carefully focused at the plane of the object but no object is present, the hollow cone of light passed through the condenser is invisible, because the objective is inside the dark base of the hollow cone. When a specimen is present, the light is deviated, or scattered, into the objective by structures on the specimen. A bright image of these details is then visible against a dark background. Because of the high contrast of the image, the system is capable of detecting extremely fine particles.

Color is seldom produced in a darkfield microscope, except in fluorescence work; thus, black-and-white films are used predominately. Since considerable light is lost in this system, a high-speed film, such as KODAK TRI-X Pan Film, is appropriate–particularly when the subject is in motion.

A darkfield microscope is an excellent tool for use in biology and medicine. It can be used effectively at high magnification to detect and photograph living bacteria. Similarly, at low magnification, whole mounts and tissue sections can be viewed and photographed. In marine biology, a darkfield microscope at very low power is used extensively for recording sea life such as algae and plankton.

Stop-Contrast Method

Darkfield illumination provides the means for the greatest enhancement of image contrast. It is especially valuable for particles, but is extremely wasteful of light. Wilska worked out a "stop-contrast," or "anoptric," method for improving the image contrast of unstained specimens. While his method was developed after the advent of phase-contrast systems (to be described in the next section), it is easier to understand, and therefore is presented first. Also, the basic scheme of manipulating "direct" and "deviated" beams is common to both systems.

The anoptral arrangement is quite similar to a brightfield system, but the image effect is one of darkfield (or semi-darkfield, as pointed out further on). A ring-shaped, "annular" slot is located near, or projected to, the condenser. This is illuminated by a solid-source lamp (ribbon filament or arc). The ring of light becomes the source for the microscope. It is focused, by the condenser and objective, near the periphery of the rear aperture plane of the objective (not in the plane of the primary image). See Figure 24.

Thus, the *potential* path of this ring of light (the direct beam) is:

- Through the condenser to the slide, where it is spread (defocused) evenly over the field, for illuminating the specimen and background.
- Directly through the background to the objective, where it is gathered back into a ring in the rear aperture plane of the objective. (Any rays going directly through a thin specimen pass through this ring to the primary focus.)
- Defocused in the plane of the primary image, where the specimen is in focus.

Finally, the ring of light is integrated by the eyepiece without structure (i.e., no bright ring appears), but the direct light from the specimen, being in focus at the primary image, is refocused.

This was specified as the *potential* path of the annular rays. However, in the anoptric system, the hollow beam is partially blocked in the aperture plane by a *semiopaque* (usually 90 percent) ring coated on the rear element of the objective. The shadow of the ring mask matches the focused aperture-plane image of the ring source, thereby allowing only 10 percent of the illumination from the source to be integrated by the eyepiece.

It should be remembered that the annular source is integrated in the slide, so that a specimen can transmit some of the direct rays to the primary plane, where they are not completely masked off. Since a transparent specimen has such low structural opacity, the direct beam through it contributes only a little

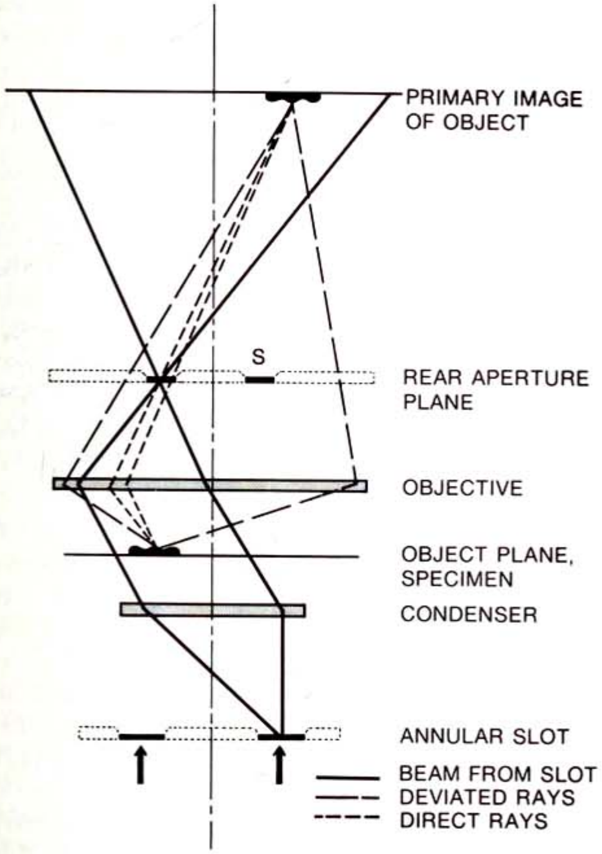


FIGURE 24—Schematic path of rays through an annular slot placed in the rear focal plane of a condenser for phase-contrast microscopy. The slot comes to a focus (S) in the rear aperture plane of the objective. From there the beam of rays is spread (defocused) over the plane of the primary image of the specimen. The object, a small detail in the specimen, transmits part of the beam (direct rays) and scatters the rest (deviated rays). Both sets of rays from the object come to a focus in the primary-image plane. If an opaque ring were placed over S, the illumination beam would be blocked from the primary image and a darkfield situation would prevail. However, the deviated rays from the object would get around the opaque ring. In the anoptric system, a partially opaque masking ring is coated on the objective. In the phase-contrast system, a transparent compensating plate, carrying a 1/4-wave, phase-changing ring, is inserted somewhere in the objective train. This changes the phase of the direct rays so that they can produce an interference pattern with the deviated rays.

to the final image detail. As in a darkfield microscope, scattered light from the specimen also forms an image. In essence, a darkfield situation is presented to the eyepiece. The background brightness depends on the degree of opacity of the annular mask.

The optical arrangement is not the same as that of conventional darkfield illumination, however. Instead, as in Kohler illumination, the source passes defocused throughout the specimen plane. When a transparent specimen is in place in a phase-contrast microscope, edges and very fine detail diffract and scatter the light. This deviates many of the rays from the specimen structures, and that is why they are not refocused into the annular ring image, where they would be masked off. Instead, they appear in the primary image (and eyepiece), just as particles do under darkfield illumination. The scattered light from such structures furnishes the "deviated" beam.

Contrast is improved partly because of this darkfield tone arrangement, but chiefly because central, direct illumination that would come up through a fully lighted condenser is not present with an annular source. Such central rays flood a field from all angles, whereas the obliquity of peripheral rays provides modeling for the specimen. This modeling appears in the deviated beam, but would be too weak to be seen with transparent specimens were a central beam present.

However, when a specimen is stained, the dye pattern is strong enough under Kohler illumination to modulate a central beam, minimizing the need for optical contrast enhancement.

In some anoptric arrangements the annulus is not blocked off. Instead, it is left clear, but the rest of the rear surface of the objective is coated—usually with a 50 percent opacity. Thus the contrast roles of the direct and deviated beams are reversed. The first mode is useful for recording specimens of low visibility such as unstained chromosomes; the second, for highly refracting, bright subjects like yeast cells.

By varying the thickness (opacity) of the coatings that do the masking, the intensity of the direct background beam and the amount of light deviated by the specimen can be adjusted. Wilska found that an even balance (equal intensities) provided the best delineation for most subjects.

Phase-Contrast Method

The phase-contrast microscope is probably the most outstanding contribution to microscopy in recent years. It can be used to produce excellent contrast effects with a wide variety of otherwise-transparent specimens. Since it permits visualization of interior details in cell structures, it has a definite advantage over the darkfield microscope. Probably its widest application is in the field of tissue culture, where it permits one to examine and photograph living, growing cells. Here, time-lapse cinephotomicrography is of definite advantage, since a specimen can be photographed at intervals timed to be synchronized with change. Subsequently, the photographs can be projected at increased rates of speed. The study and photomicrography of living blood specimens by phase contrast also becomes possible, providing a means of visualization previously impossible.

Phase Optics: The phase-contrast method was introduced by Zernike. Fundamentally, the procedure involves a direct and a deviated beam, as in the anoptric system. (See Figure 24.) The difference is that the masking and balancing of the beams are accomplished by phase-interference modulation, rather than by opacity modulation.

A word on this interference phenomenon may be helpful here. When a ray of light from a single point source is split in two and passed through (refracted) a transparent medium, the two rays can be recombined without interference. But if one passes through a medium of different refractive index, it is speeded up or slowed down, depending on the index. Then the two, when recombined, may be out of phase. If so, interference occurs and the recombined beam is not as intense as the original. To appreciate this, visualize the two split rays as waves traveling side by side. If neither one is altered, they can combine "in phase." Peaks will meet peaks and valleys will meet valleys; there is no destruction of intensity. But if one component is altered in velocity, the waves may no longer match in configuration. When the configurations differ by less than a wavelength, the waves are out of phase and their recombination results in a loss of intensity. When they are out of phase by 1/2 wavelength, peaks will meet valleys and the rays will cancel each other out, extinguishing the beam.

In addition to refraction and retardation by media, diffraction at edges and scatter from very fine details can also change the configuration of the waves. Diffraction is largely responsible for the edge effects seen in phase-contrast microscopy.

In the phase-contrast system, an annular source, again, is utilized (although linear and crossed slits can be employed). But instead of an annular density mask behind the objective, a "1/4-wave" ring is used. This retards a wave by 1/4 wavelength. To form this mask, a transparent medium is coated in a ring on a "compensating plate," which is also transparent. The plate is positioned behind the objective in its aperture plane, where the annular source comes to a focus. The 1/4-wave ring is aligned with the focused ring source.

As in the anoptric system, the beam from the annular source is defocused in the slide and in the primary image. But its light, not being masked with an opacity, can be integrated by the eyepiece. However, neither the eye nor the film can detect the 1/4 wave change in phase.

As before, the specimen, in the defocused direct beam, deviates much of the light, depending on index, thickness, and fine structure. Again the deviated light goes to the eyepiece via the primary image. However, the deviated rays have been changed in phase by the specimen. The change plays a vital role in forming a high-contrast image, even though it may be as small as 1/20 wavelength.

A direct beam is passed by the background. Some direct light is not deviated by the specimen. Thus, when the illumination reaches the specimen, it is split into direct and deviated rays by the specimen itself. The direct rays go into the 1/4-wave ring. But while the direct part through the specimen has no more opacity modulation than it does in the anoptric system, it now does have an added phase modulation produced by the ring. The deviated rays also have a phase change dependent, in degree, upon the diffraction of the specimen.

When the beams of direct and deviated rays from the specimen are focused in the primary image, interference occurs between the two recombined, specimen-image components, and between edges and background. The amount of intensity reduction in any given structure depends upon the phase difference of the two rays imaging that structure. When this is 1/2 wavelength, maximum reduction (darkness) occurs.

The brightness of the background (in a given setup) is always the same tone, corresponding to a medium gray in photography. But the brightness of the specimen image relative to this tone can be altered by several means. Phase plates and mounting media control this aspect.

Some phase control is done with an annular compensating plate. As in the anoptric system, the contrast roles of the two beams can be reversed. In the phase method, the compensating plate can have a ring ground thinner than the rest of the plate, rather than a 1/4-wave coating. Then the direct beam is advanced 1/4 wave instead of being retarded, because of the shorter path through the plate. With a retarding phase plate, if the retardation of the medium and the phase ring add up to an amount that is not sufficiently revealing, a compensating plate with an advancing ring can be substituted. This will make a 1/2-wave difference in the configurations of the waves going through the phase-ring area.

Other plates are sometimes used to alter the phase of the deviated beam by specific amounts. This is done because it has been found that the best rendition of detail usually occurs when most of the details in the two beams are about 1/4 wave out of phase. Also, for good results, the brightness of the two components of the specimen image should be equal. So plates coated with thin opaque metallic films can be used to balance the intensity of the beams. Plates are selected from knowledge of the indices of medium and specimen, or quite often by trial.

A somewhat confusing point in the nomenclature arises from the difference between the designations of European and American equipment manufacturers. The first group defines a "positive" phase plate as one which advances the direct beam, and a "negative" phase plate as one which retards this beam. The opposite is elected by the second group. It is better not to mix these terms with "contrast" designations. The reason is that a given phase plate can produce bright or dark contrast images, depending upon whether the index of refraction of the specimen is higher or lower than that of the medium. In general, designations are based on the specimen having the higher index. But an objective marked "dark contrast" can yield the opposite effect when the medium is changed. It is obvious that the photomicrographer should fix his mind on what his particular equipment does, rather than go entirely by what it is called.

The image appearance, regardless of the optical elements used to modify it, is quite indicative of the type of contrast that has been obtained. When the specimen is generally lighter than the background, "bright-phase" contrast exists; when darker, "dark-phase" contrast exists. The outermost fringe, or halo, between the specimen and background is black in the first instance and white in the second. (Figure 25.)

Effects of Mounting Media: Contrast can be manipulated in degree and "sign" to a practical extent by selection of the medium in which the specimen is mounted. If the refractive index of the medium is too close to that of the specimen, very little contrast will result. It is suggested that the medium differ sufficiently in refractive index to provide adequate contrast for both visual examination and photomicrography. When tissue specimens are examined, for example, a medium other than balsam is needed. Balsam is a common mounting medium, but its refractive index is very much like that of unstained tissue. A medium such as glycerine, for temporary mounts, or Diaphane and white Karo syrup for permanent mounts, will provide much better contrast. The refractive index of balsam is about 1.53; while Diaphane and glycerine have a lower index, about 1.47. (See mounting media, Table 3, page 24.)

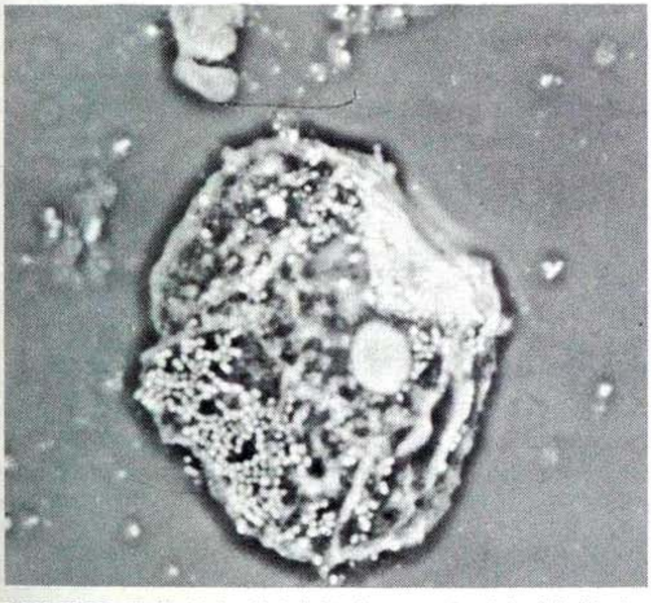


FIGURE 25—Epithelial cell, bright phase contrast. In this illustration, note the dark halo present around the cell and the artifacts. In dark phase-contrast records, the halo is almost white. KODAK EKTAPAN Film.

Filtration: In phase-contrast photomicrography, a green filter is commonly used in conjunction with a black-and-white film. The green filter is appropriate because phase objectives are most often corrected optically for green light. An excellent filter for this purpose is KODAK WRATTEN Filter, No. 58. When a somewhat deeper green is desired, use a KODAK WRATTEN Filter No. 61.

Color films are not normally used, since the phase-contrast method is most frequently used with colorless, or almost colorless, images of low original contrast. Moreover, optimum phase-contrast effect is obtained with a single color, normally green. Possible exceptions to this may be photomicrography of very lightly stained objects or photomicrography with polarized-light. A green-sensitive, black-and-white film of moderate contrast is quite suitable for phase-contrast photomicrography. When sheet film is used, KODAK EKTAPAN Film 4162 (ESTAR Thick Base) is highly recommended. This film has very fine grain and wide development latitude, with moderate speed. When a higher speed film is necessary in order to stop the motion of living specimens, KODAK ROYAL Pan Film 4141 (ESTAR Thick Base) or KODAK ROYAL-X Pan Film 4166 (ESTAR Thick Base) is indicated.

For 35 mm still photomicrography, KODAK PLUS-X Pan Film, KODAK TRI-X Pan Film, and KODAK PANATOMIC-X Film are recommended. If higher contrast is desired, KODAK Photomicrography Monochrome Film (ESTAR-AH Base) SO-410 and KODAK High Contrast Copy Film 5069 are applicable. These films have extremely fine grain and have proven excellent for this application.

In cinephotomicrography, 16mm EASTMAN PLUS-X Negative Film 7231 is recommended when extremely fine grain is desired; EASTMAN 4-X Negative Film 7224, when extremely high emulsion speed (EI 500) is essential.

Interference-Contrast Methods

There are several other types of microscopes that utilize the interference effect. The most useful ones for use in general fields are those based on the interference-contrast principle. The method does not yield the contrast enhancement of a phase-contrast microscope, but it does eliminate most peripheral and structural edge halos, thereby delineating fine detail. It also introduces color effects and gives a three-dimensional appearance of relief. (See Plate II-E.)

Apart from the fact that the condenser is fully illuminated, the basic difference in the optics of the two systems is this: In the interference-contrast microscope, it is the optical system that produces the two interfering beams; it is not the specimen, as in the phase-contrast method. The interference-contrast beams are called the "object beam" and the "reference beam." The first carries the image, and the second is either a homogeneous or an asymmetrical beam. Interference takes place between two types of beams, not between two image components, as in the phase-contrast system.

Again, several analogies may be helpful. In the phase-contrast system, the configurations of the two beams are like the wakes of two boats merging courses on a smooth lake. The interference pattern they create is sharpest when the boats have the same size and speed (balanced beams). In an interference-contrast system, the wake of a single boat interferes with waves on a rough lake. Balance is best when the lake is no rougher than the wake of the boat. To apply the analogy to the Nomarski system (to be discussed), the wakes of two boats traveling side by side interfere to produce the pattern; the wake of the boat studied has the wake of the other asymmetrical to its course.

Once the object and reference beams are split, they are separate in the plane of the specimen. They are recombined for interference before they reach the eyepiece. There are two commonly used methods (due to Smith) for affording this separation. They are the "double-focus" system and the "shear" system.

In the first, both beams are focused by the condenser along the optical axis of the microscope. The object beam is focused in the plane of the specimen. The reference beam is focused above or below the slide and forms no structured image. In effect, the specimen is seen through the even blur of the reference beam. The two beams are displaced vertically. The wanted detail is imaged and interferes with the same detail out of focus.

In the shear system, both beams are focused in the plane of the specimen, but they are optically displaced laterally. In effect, only the subject beam illuminates the specimen—the reference beam is moved aside for subsequent recombination below the eyepiece. This beam should pass through a relatively clear area of the slide in order to minimize the imaging of unwanted detail, which would intrude on the subject detail. Thus this system is not as suitable as the double-focus method of studying tightly packed slides.

Nomarski Optics: A commonly used interference system for all powers of objectives was worked out by Nomarski. It employs a modified shear system. The illumination from the lamp passes through the following components (see Figure 26):

- A polarizer below the condenser produces a beam of plane-polarized light.
- A Wollaston prism splits the beam in two before it enters the condenser. It also rotates the planes of polarization of the two beams so that they are mutually perpendicular. They cannot interfere where they cross at various foci in the optical train.
- After passing through the condenser, the slide, and the objective, the beams are recombined by another Wollaston prism with oppositely cut faces. However, they still cannot interfere.
- A diagonally oriented polarizing analyzer plate circularly polarizes (see page 67) the two beams so that they can interfere before entering the eyepiece to form the interference contrast image.

Operational Details: Photography of interference-contrast images is similar to that used for brightfield illumination. However, it may be necessary to reduce the aperture diaphragm to obtain more even illumination. If this is done, the resolving power of the objective will decrease. Consequently, the best aperture setting is largely dependent on the specimen itself, and some experimentation will be necessary to obtain the best compromise.

The Nomarski system is very efficient in that it usually permits a full aperture. It is able to resolve finer structure than the phase-contrast method. However, the Nomarski image may be somewhat deceptive with respect to topography, depending upon whether the object beam or the asymmetrical reference beam dominates the image. For this reason, the photomicrographer should have some idea of the surface contours of the specimen. (For example, notice the change in appearance that occurs when Plate II-E is viewed upside down.) Then, by means of a rotating stage (which he ought to employ), he can orient the slide until the image conforms. In this way the full benefit of the system for delineating transparent subjects can be obtained.

Francon System

Certain three-dimensional specimens in the metal, plastics, and glass industries and in crystallography and metallography can be examined by reflected interference with a system developed by Francon. A polarized-light microscope is adapted for axial illumination. Instead of Wollaston prisms, Savart plates, which are double, flat plates of quartz crystal suitably cut, serve to produce divided beams. The degree of separation of the beams depends upon the thickness of the Savart plates chosen and can be made quite spacious. (See Plate II-E.)

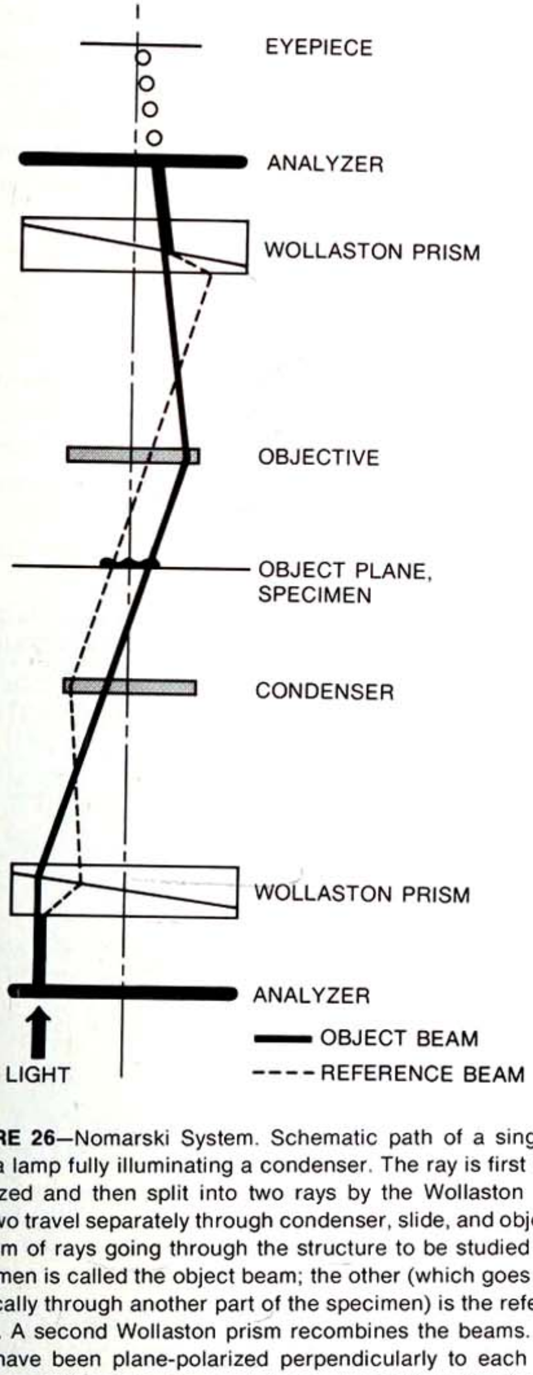


FIGURE 26—Nomarski System. Schematic path of a single ray from a lamp fully illuminating a condenser. The ray is first plane-polarized and then split into two rays by the Wollaston prism. The two travel separately through condenser, slide, and objective. A beam of rays going through the structure to be studied in the specimen is called the object beam; the other (which goes asymmetrically through another part of the specimen) is the reference beam. A second Wollaston prism recombines the beams. Since they have been plane-polarized perpendicularly to each other, they cannot interfere. So a polarizing element (analyzer) with its plane of polarization at 45 degrees to each of the incoming beams is introduced. The analyzer circularly polarizes each beam so that they can combine to form interference patterns.

The films and processing conditions recommended for the brightfield illumination technique on page 32 apply in interference-contrast microscopy. Exposure settings should be determined experimentally.

Polarizing Method

Polarized light has many valuable properties that merit understanding by the photomicrographer. Most illumination sources for microscopy emit beams of heterogeneous light—the waves are vibrating in all directions perpendicular to the axis of propagation, like paper streamers tied to the guard on an electric fan. If the fan were brought close to an open venetian blind, only those streamers vibrating in the horizontal plane would pass through the slits. If the blind were hung sideways, only the vertically vibrating streamers would pass through. Two blinds, crossed in this fashion, would block all the streamers. Polarizing media present optical slits that perform in a way somewhat like these blinds. They pass half of the light "plane-polarized" according to the orientation of the slits. The other half is absorbed.

Transparent "isotropic" objects pass plane-polarized light unchanged. "Anisotropic" materials, such as crystal quartz, can split a plane-polarized ray into two rays. A Wollaston prism, made up of two opposed quartz crystals, serves to rotate the planes of the two ray components perpendicular to each other. And of course, an oppositely oriented prism can recombine the two rays. (See Figure 26.)

In the Nomarski microscope, both beams are circularly polarized as they recombine. They are able to create interference effects, which two rays cannot do when plane-polarized perpendicularly to each other. This is accomplished in the following way: In the path of a plane-polarized ray, when another polarizer is placed with its slits (plane of polarization) at 45 degrees to the vibration of the waves, the ray becomes "circularly" polarized. (A circularly polarized beam should not be confused with a heterogeneous beam.) If an increment of plane-polarized light can be visualized as a sheet of paper moving (parallel to the optical slits) along the beam, then an increment of circularly polarized light would be a sheet of paper edgewise spinning around its axis of propagation as it moves along the beam. A heterogeneous increment, however, could be represented as a moving book opened up so wide that the covers are touching.)

In the ordinary polarizing method, the microscope system is quite simple—a "polarizer" is placed below the slide and an "analyzer" at the eyepiece. These are mounted with their slits perpendicular to each other; they are set for "extinction." Now, the object itself, when it is anisotropic, plays a role somewhat like that of a Wollaston prism. It splits and rotates the plane-polarized rays from the condenser by a small amount, so that when they reach the perpendicularly oriented analyzer, they are not extinguished by it. But rays going through an isotropic object are so changed; thus they are extinguished. One obvious benefit is differentiation of anisotropic and isotropic areas within a transparent specimen, say, in a thin slice of rock for petrographic study.

But there is another, better-known effect produced by the method—interference colors. These are created by a growing anisotropic crystal, for example. This subject consists of structures of varying thickness. A crystal is a medium which retards the phase of rays passing through it, and an anisotropic crystal also modifies the polarization. Retardation and the degree of anisotropic splitting depend upon thickness and wavelength; hence, the spectral colors in white light are not all affected alike. When two adjacent rays pass through a given spot in the crystal and are split by it, they are close enough for interference, and this may cause the blue components to augment each other. In another spot, of different thickness, a red color may appear. Because of the concomitant rotation of their planes of polarization, these colors can pass through the analyzer and form the image pattern. Rays through the background of the slide are not rotated and hence are extinguished, preventing the background from flooding the image.

A "tint" plate is a thin lamina of a birefringent substance like mica. The thickness is accurately controlled so that the effective retardation is a specified fraction of a wavelength. It has a transmission axis of maximum refringence. This axis can be oriented at various angles to the plane of the polarized beam for controlling the region of the spectrum at which maximum wavelength augmentation takes place. Also, its retardation can be added to that of a weakly birefringent specimen for an enhanced image.

Some isotropic materials can become anistropic under strain, the degree depending upon the stress. Such objects also exhibit interference patterns in color when white illumination is employed. Monochromatic light yields dark and bright strain lines.

Very striking effects can often be obtained with a brightfield microscope equipped with polarizing filters. Some transparent substances, principally certain chemical crystals, have an optical property called "birefringence," or "double refraction." When such substances are viewed in a microscope between crossed polarizers, they may appear bright or even colored against a dark background. Place one polarizer in the illumination beam and the other somewhere behind the objective, usually on the eyepiece.

The formation and growth of birefringent chemical crystals is a fascinating subject that can be photographed on color film, with either a still or movie camera. A low-power microscope is sufficient. Crystals are formed by dissolving a chemical in distilled water, placing a drop of the solution on a microscope slide, and spreading the solution thin. As the water evaporates, the crystal growth begins. Either of the polarizers can be rotated to produce the crossed position, which results in a dark background on which the colored crystal pattern forms. Sodium thiosulfate crystals (commonly known as "hypo") work extremely well for this study. A bright light source is generally needed. (See cover illustration.)

Very slight birefringence can be detected and photographed by placing a sensitive tint plate between the polarizer in the light beam and the analyzer. In this case, the background will be colored; the exact color depends upon the orientation of the plate.

Other subjects which show polarization effects are rock sections ground thin for petrography, fibers, hairs, starches, and many components of plant and animal tissues.

Stereo Method

When one looks at any object, the image recorded by the optical system, in conjunction with the coordinating facility of the brain, has a three-dimensional effect. This effect is the result of two different views of the object, separated by an angle dependent upon the interpupillary distance of the individual, and then interpreted as one image. This is the principle of the Greenough binocular dissecting microscope, designed in 1897 for low-power work. In this type of microscope, each eye is provided with its own complete microscope. The paired objectives are mounted together at the approximate angle of binocular vision: 15°. The rays from the objective pass through a Porro prism, which produces an image that is not inverted. The prisms in the paired objectives are similar to those used in field binoculars. This microscope has been modified and improved in recent years. The objectives have been incorporated into a drum, or sliding nosepiece. The use of wide-field oculars and prisms produces a large, flat field of view, with the range of magnification from ×3 to ×100. This type of microscope has the advantages of great depth of focus, long working distance, large, flat fields, and simplicity of operation.

The "stereo" microscope, as it is commonly called, has become invaluable in industry, medicine, teaching, or wherever a microscope of low power is needed.

Most newer stereo microscopes now have zoom capabilities and a maximum magnification of ×200. The use of stereo microscopes varies from general scientific investigation in all fields to production examination in quality control. The most important use in relation to photomicrography is in examining the specimen to determine the best area to photograph. This is especially necessary in photomicrography of biological specimens—where dissecting, staining, and selective mounting are performed.

This microscope has several limiting factors when specimens are to be photographed through one of the body tubes. The principle limiting factor to producing quality photomicrographs with stereo microscopes is the low N.A. of most objectives. Additionally, since only one objective, at a slight angle to the specimen, is used, the depth and resolution typical of stereo views is not recorded on film. Many microscope manufacturers have accessories to correct this situation. Paired stereomicrographs can be made in several ways that are described in *The Practical Use of the Microscope* by G. H. Needham. The best film to use is KODACHROME 64 Film (Daylight) or KODACHROME II Professional, Type A, depending upon the color temperature of the light source. These films have fine grain and good resolving power.

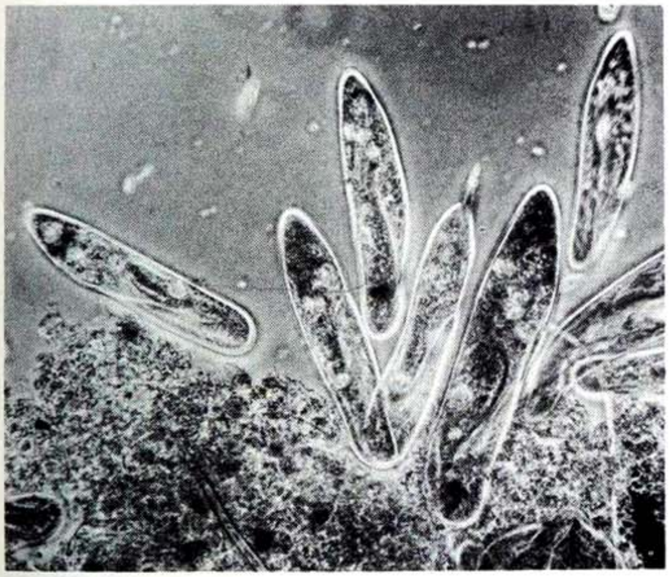


FIGURE 27—Paramecia photographed at low power in a binocular microscope.

SPECIALIZED APPLICATIONS

Fluorescence Photomicrography

Some materials will emit light of a longer wavelength when excited by short wavelengths of radiation. This phenomenon is called "fluorescence." Either ultraviolet or blue light is often used as an exciting radiation to produce visible light of longer wavelength. If a substance does fluoresce, the effect is called either "primary fluorescence" or "autofluorescence." Some materials do not fluoresce by themselves but can be impregnated with chemicals, such as certain dyes (see Table 5, page 33), that will fluoresce. Dyes of this type are called "fluorochromes"; the effect in the original material is called "secondary fluorescence." For example, when excited with ultraviolet, chlorophyll will fluoresce with a deep red color. When stained with dilute acridine orange, human epithelial cells will glow an orange-red under ultraviolet radiation. (See Plate II-D, page 38.)

Fluorescence microscopy and photomicrography have acquired great importance in recent years in the field of exfoliative cytology. A very appropriate application is in early detection of cancer in smears, exudates, and tissue sections. Fluorescence techniques are used to advantage in other fields of medical and biological research also.

Light Source: A very efficient light source for most fluorescence work is the high-pressure mercury-vapor lamp, which emits very bright radiation in both ultra-violet and short-blue wavelengths. When a light source having a continuous visible spectrum as well as ultraviolet is needed, the xenon arc serves the purpose very well. Most fluorochromes are excited by ultraviolet and produce fluorescence somewhere in the visible spectrum. The fluorescein dyes commonly used in antibody techniques have a maximum absorption for long blue wavelengths, however, and require a continuous source, such as a xenon arc.

When a substage mirror is used, one with an aluminized surface should be selected. The reason for this is that silver, often coated on these mirrors, is a poor reflector of ultraviolet radiation.

When ultraviolet radiation is used to produce fluorescence, such as with a mercury arc, an exciter filter is needed in the light beam to transmit this radiation freely and to absorb visible light not needed in producing fluorescence. KODAK WRATTEN Filter No. 18A (glass only) has a high transmittance for the 365 nm (ultraviolet) line of the mercury-vapor spectrum. It absorbs all visible light. A barrier filter is also necessary. This type of filter should absorb ultraviolet, such as that transmitted by the specimen, and transmit visible radiation freely, including fluorescence colors. The selection of an efficient barrier filter can be critical. A KODAK WRATTEN Filter, No. 2B, 2A, or 2E, can be used for this purpose. Although each filter will completely absorb ultraviolet, the difference is in their absorption of short-blue wavelengths. If in doubt, the No. 2A Filter is suggested.

Fluorescein Dyes: In a fluorescent-antibody technique, where fluorescein dyes are used as fluorochromes, secondary fluorescence usually occurs in the green at about 540 nm. Maximum absorption of these dyes is at 480 to 490 nm in the blue region of the spectrum. A filter which transmits blue freely is normally employed as an exciter filter. KODAK WRATTEN Filter No. 47B is suggested. A barrier filter for this application must transmit wavelengths longer than blue and must absorb blue completely. A KODAK WRATTEN Filter, No. 12 or No. 15, will suit this purpose. Both filters are yellow and will transmit the green fluorescence color freely.

When fluorochromes are used to stain specimens, a certain amount of short-blue "autofluorescence" occasionally occurs also, particularly with tissue sections. This autofluorescence often must be absorbed in order not to degrade the color of the secondary fluorescence produced by the fluorochrome. Both the No. 2A and No. 2E Filters have absorption for short-blue radiation, the No. 2E having the greater absorption in this respect.

A barrier filter must be used behind the objective. If it is not, the residual ultraviolet will record as blue on color film and will degrade all fluorescence colors. A barrier filter of appropriate size can often be used directly behind the objective in the microscope body tube, or a smaller-size filter can be placed in or on the eyepiece of the microscope.

The selection of a mounting medium is also important. A medium must be chosen which has little or no autofluorescence. Autofluorescence of a mounting medium may be either pale blue or pale green in color and will degrade fluorescence colors. Temporary mounts can be made with either pure glycerine or Cargille's Immersion Oil, Type A, or Crown Oil (obtainable through scientific supply houses). These have very low fluorescence. Care should be taken when handling these oils, since both contain high percentages of toxic polychlorinated biphenyls. If glycerine containing such impurities as "acrolein" is used, a light greenish autofluorescence may occur. Most permanent mounting media also produce autofluorescence; therefore, they should not be used in fluorescence work.

The pale-blue autofluorescence of a mounting medium can be absorbed to some extent by either a barrier filter which absorbs some blue or a pale-yellow KODAK Color Compensating Filter, such as a CC20Y. Pale-green autofluorescence can be neutralized by a pale-magenta color-compensating filter, such as a KODAK Color Compensating Filter CC20M. Heavier filters may also absorb some of the desirable fluorescence color. Quite often the KODAK WRATTEN Filter, No. 2E will absorb ultraviolet as well as the pale-blue autofluorescence of a mounting medium.

Darkfield illumination is highly recommended for both fluorescence microscopy and photomicrography. By this technique, the darkest background is achieved so that fluorescence colors stand out brightly. As previously stated, oil-immersion darkfield condensers are most efficient for medium and high power. Take great care that no bubbles are introduced into the oil and that the microscope slide is of the correct thickness for the condenser in use. A darkened room is highly essential to efficient darkfield work in fluorescence microscopy in order to exclude extraneous light, even from the slide surface.

Even though a fluorescent image may appear bright to the eye, long exposure times are often necessary in photomicrography. Color films, of course, are suggested in order to record the image in color, as it appears to the eye. Therefore, a high-speed film—such as 35 mm KODAK High Speed EKTACHROME Film, (Daylight)—will minimize exposure time and will record most fluorescence colors with reasonable accuracy (see Plate II-D on page 38). Daylight-type film is suggested because of its balanced sensitivity to red, green, and blue. This film can also be specially processed for higher speed (see page 34). Other daylight-type, color-reversal roll films such as KODACHROME 64 or KODAK EKTACHROME-X Film, although of lower speed, can also be used to record particular fluorescence colors.

Ultraviolet Photomicrography

Since the limit of resolution attainable in photomicrography depends upon the wavelength of light, the highest resolution is obtainable with ultraviolet. It is possible to make photomicrographs in the near ultraviolet (365 nm) by using a conventional microscope with a glass lens. Apochromatic objectives perform best. Optical glass, however, will not transmit ultraviolet radiation below a wavelength of about 310 nm. In order to transmit the shorter wavelengths, a microscope equipped with fluorite optics or reflecting optics must be used. One of the principal advantages of this type of microscope is its ability to focus an image in visible light and to keep the image in focus throughout the ultraviolet region. In conjunction with this microscope, a light source such as a high-pressure, mercury-vapor lamp is needed to provide radiation in the far ultraviolet. For photomicrography, a "monochromator" is also required in order to isolate a particular wavelength or narrow band of wavelengths in the ultraviolet.

Quartz optics and other types of microscopes and illumination, as well as a means for focusing the invisible ultraviolet radiation, are discussed fully by Loveland (1970).

Photomicrographs in the near ultraviolet can also be made with this ultraviolet microscope. It provides a higher degree of resolution than is obtainable with a conventional microscope. In addition, the ultraviolet microscope allows one to study the selective absorption of ultraviolet by living cells, tissues and fine particles.

What types of films are used in ultraviolet photomicrography? All photographic emulsions have an inherent sensitivity to blue and ultraviolet. Sensitivity in the ultraviolet actually extends far into this region, but the response of a film or plate is somewhat limited, due to absorption of ultraviolet by gelatin, the medium in which the silver-halide crystals of the emulsion are suspended. For long wave ultraviolet photomicrography, almost any film or plate can be used to record the image. However, only black-and-white films need be considered, since color film has no advantage. In 35 mm ultraviolet photomicrography, KODAK PANATOMIC-X Film is especially recommended because of its extremely fine grain which permits excellent enlargements. When a higher contrast is needed, KODAK High Contrast Copy Film 5069 may be used. If sheet film is to be used, a very fine grain film of medium contrast should be the first choice. Films such as KODAK EKTAPAN Film 4162 (ESTAR Thick Base) and KODAK PLUS-X Pan Professional Film (ESTAR Thick Base) are quite suitable.

Images formed by ultraviolet photomicrography are low-contrast. Therefore, if a high-contrast film is not used, medium- to high-contrast development can achieve satisfactory results. To obtain medium- to high-contrast development, either extend the recommended development time or use developers that produce higher contrast. It should be noted that the color-blind and orthochromatic films (that is, the blue- and blue-green sensitive films) generally provide a higher contrast than panchromatic materials.

When higher sensitivity to ultraviolet is needed, or when sensitivity is desirable in medium-wave and short-wave regions of the ultraviolet, it may be necessary to resort to special emulsions. Eastman Kodak Company can supply information about special materials that are available. Also, information about these products can be found in *KODAK Plates and Films for Scientific Photography* (Kodak Publication No. P-315) and in *Ultraviolet and Fluorescence Photography* (Kodak Publication No. M-27). These Data Books can be obtained through photographic dealers or from Eastman Kodak Company by direct order.

Infrared Photomicrography

Most of the subjects examined through a microscope transmit light to some extent, even if treated with stains. Some subjects, however, are relatively opaque when viewed through a microscope; few, if any, details are visible. Increased visibility can often be obtained by the use of infrared, the long wavelengths beyond the visible spectral range. Infrared photomicrography is especially useful in the field of entomology. Many insects have chitinous and dark-pigmented structures that are opaque to visible light but are penetrated freely by infrared radiation. Heavily stained specimens, many textiles, forged and altered documents, dark-colored crystals, and many other subjects can be photographed with infrared radiation. With a fast, color infrared film, photography in the infrared can be used for many applications. Some of the most common applications are in plant pathology, pollution control, criminology, and histology. Infrared color photography has been used for differentiating biologic pigments, tissue structures, and inclusions, as well as for finding new criminal detection methods.

Because of the long wavelength of infrared radiation and because of lens aberrations, in that region, the infrared image cannot be as sharp as in ordinary photomicrographs. The benefits comes from the penetration of many visually opaque specimens. (See Figure 28.)

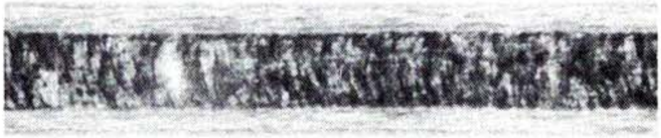


FIGURE 28—Dense animal hair photographed with infrared-sensitive black-and-white film and a KODAK WRATTEN Filter No. 87.

A color film used for infrared photomicrography is KODAK EKTACHROME Infrared Film; it is available in 20-exposure rolls for 35 mm cameras and 100-foot rolls for 16 mm cameras. This film is a false-color reversal film that was originally designed for camouflage detection by aerial photography. It is balanced for daylight-type exposures, (or for exposures by electronic flash) with a KODAK WRATTEN Filter, No. 12 (yellow), over the camera lens.

For use in infrared photomicrography, Kodak black-and-white infrared films with broad infrared sensitivity, are available in sheets or in 35 mm rolls. One of these highly sensitive films, KODAK High Speed Infrared Film 2481 (ESTAR Base), is available in 16 mm and 35 mm, 100-foot rolls as well as in sheet form [KODAK High Speed Infrared Film 4143 (ESTAR Thick Base)] for applications requiring high speed film. Twenty-exposure magazines of KODAK High Speed Infrared Film are also available. Also, special black-and-white emulsions with sensitivity to about 1150 nm can be obtained from Eastman Kodak Company. More information about these films may be found in *Applied Infrared Photography* (Kodak Publication No. M-28) and *KODAK Infrared Films* (N-17) available through photo dealers.

Since normal exposure-meters have low sensitivity in the infrared, exposure times are usually determined by trial. It is common practice to make a series of exposures, either on sheet film (by withdrawing the dark slide by definite amounts and varying exposure time) or on 35 mm roll film (by making several exposures of one frame each).

However, before making infrared photomicrographs, test film holders and plateholders, including the draw slides, to make certain that they are opaque to infrared radiation. Failure to do this may result in fogged emulsion and complete deterioration of image contrast. Processing recommendations for infrared films are included with the materials.

Because infrared photography is quite specialized, it is not feasible to go into details here. Instead, the applications have been given. Also, the basic requirements have been outlined so that you can see that there is no particular complexity involved. Nevertheless, certain careful but straightforward procedures are needed in focusing the photomicroscope. When infrared color film is employed, special attention must be paid to light sources and color balancing with filters. Those who wish to carry on the technique are referred to Kodak Publication M-28.

Photomicrography of Chromosomes

The study of human chromosomes is of widespread interest in cytogenetic laboratories throughout the world. Since photomicrographs of chromosome preparations must always be made for karyotyping, it is of great importance that the recorded image be of high quality in order to provide the most information. (See Figure 29.)

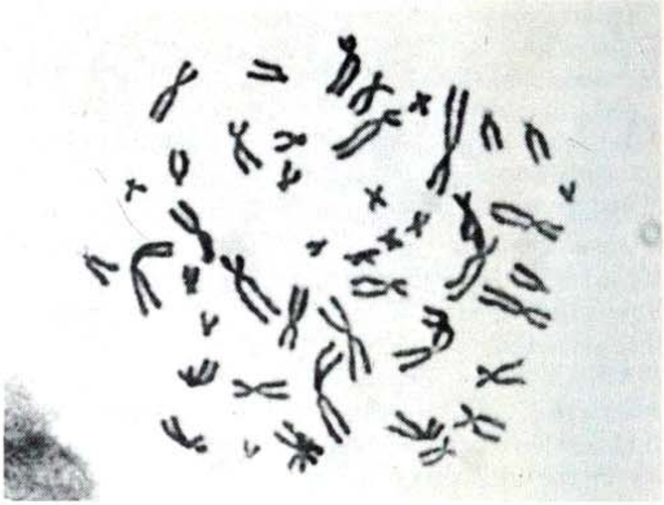


FIGURE 29—Human chromosomes. KODAK PLUS-X Pan Film (35 mm). KODAK WRATTEN Filter, No. 61.

Basically, chromosome preparations are usually made from blood cultures in which the arrest of cell division is accomplished with colchicine at the metaphase stage of the mitosis. Smears are then made on microslides and are stained to produce contrast. Aceto-orcein and Giemsa staining are quite common. The preparation is viewed and photographed, usually with a green filter, both for additional contrast and for high image quality. Because of the minute size of chromosome, a very efficient microscope with high-quality optics is essential. A bright light source is also a necessity because the photographic materials used often have high contrast but low speed.

Several 35 mm high-contrast black-and-white films used for the photomicrography of chromosomes are KODAK High Contrast Copy Film 5069 and KODAK Photomicrography Monochrome Film (ESTAR-AH Base) SO-410. Both of these films possess extremely fine grain and high resolving power. They will produce excellent results for this application. KODAK Photomicrography Monochrome Film also features variable contrast, depending upon processing conditions.

When a sheet film is to be used, KODAK Contrast Process Ortho Film 4154 (ESTAR Thick Base) has proven suitable for the purpose. Contrast may be too high when the recommended developer is used. For suitable contrast, the use of KODAK Developer DK-50 (diluted 1:1 with water) is suggested. Development time is 7 minutes at 20 C (68 F) for tray development, or 8 minutes for tank development.

The negatives are printed on single-weight, high-contrast photographic paper so that the individual chromosomes can be cut out for assembly as a karyotype. When unstained chromosomes are to be photographed for study rather than for karyotyping, the stop-contrast method (see page 62) is recommended.

Photomicrography of Autoradiographs

Autoradiography is a technique by which radioactivity is detected using photographic materials. Special photographic emulsions with high sensitivity to charged particles are employed to trace the path of radioactive-labeled substances in metabolic studies of plants and animals. Also, it is useful in studying cells, nuclei, and chromosomes in which the uptake and location of tracer substances in the various stages of cell division can be recorded or quantitatively evaluated. For further information on autoradiographic techniques, see, for example, Rogers, A. W., "Techniques of Autoradiography," Second Edition, Elsevier Press, New York, 1969.

The contact image produced on a film or plate by an object that contains a radioactive substance is called an autoradiograph. In practice, the specimen section on a microscope slide is covered with the photographic emulsion which, after exposure and processing, contains the silver grains of the autoradiographic image.

When making a photomicrograph, it is preferred that both the specimen image and the autoradiographic image be in focus together so that the information in each image can be correlated. At low magnifications the available depth of field may be sufficient to produce both images in focus at the same time. Standard photomicrographic techniques as previously described in this book can therefore be utilized. At higher magnifications–generally utilized in autoradiographic evaluations of biological specimens–both images must be separately focused for comparative evaluation. A double exposure technique, then, is required to obtain both pieces of information in focus in the same photomicrograph. Simply determine the proper exposure for one of the images and also determine the correct focus position on the micrometer scale for each image. Use one-half the exposure time to photograph each image at its determined focus position.

Photomicrographs of autoradiographic specimens may be made in either black-and-white or color. The black-and-white films that can be used are KODAK High Contrast Copy Film 5069 and KODAK Photomicrography Monochrome Film SO-410, which are contrast-dependent upon development. The color film that can be used for normal exposures is KODAK EKTACHROME-X Film; for low lighting conditions, KODAK High Speed EKTACHROME Film (Daylight) can be used. Color films can be processed by the user or can be sent to a color-processing station. (See "Processing Service for Increased Speed," page 34.)

Metallographic Photomicrography

Metallography is the science of studying, interpreting, and recording details of the physical structure of metals and other opaque surfaces. The preparation of samples for metallography necessitates their mounting, polishing, and etching. This science differs radically from other branches of microscopy. It involves many different metals and alloys, both ferrous and nonferrous. These samples may range from extremely hard to very soft. The preparation of each requires its own technique; however, all specimens examined or photographed that are opaque must be illuminated by incident light. The light, of course, comes from or near the optical axis of the microscope. A focused beam can be directed axially (see Figure 31), with the beam-splitting mirror below the objective in very low-power work. There is not enough space for this mirror when a higher-power objective is used. Thus, a special illuminator that incorporates a mirror and holds the objective is used. The objective serves as a condenser. Provision is often made for decentering the beam for oblique incidence, when recording surface irregularities rather than the specular appearance of the object. Most metallographic microscopes have "built-in" vertical illuminators and an attachable light source.

The metallographic microscope differs from an ordinary microscope, in that it includes a focusing stage and that the vertical illumination stays constant. The Le Chatelier type of inverted microscope is most commonly used in metallography.

With this microscope, the specimen is held perpendicular to the optical axis of the objective by placing it face down on the stage. The stage can then be moved up or down by both coarse and fine focusing adjustments. The objective is held in a vertical position beneath the stage (Figure 30). Alignment of the

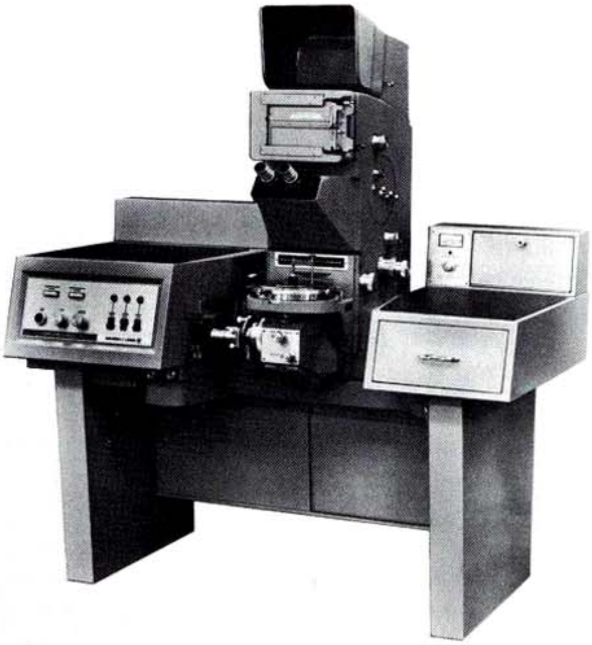


FIGURE 30—The metallographic microscope allows photographs to be taken of surface details of many opaque specimens. (Photo courtesy of Bausch and Lomb, Inc.)

camera, microscope, and illuminator is comparatively permanent, so speed and accuracy are assured for production work. Various light sources are adaptable for use with this type of microscope.

Some types of inverted microscopes include built-in facilities for polarized-light and darkfield work, as well as the conventional brightfield arrangement. Accessories are available for phase contrast and for interferometry. Inverted metallographic microscopes are made commercially by several manufacturers, both foreign and domestic.

More information about the factors involved in metallography can be found in Kodak Publication No. P-39, *Photomicrography of Metals,* available from photo dealers.

Photography of Microelectronic Circuits

The microelectronic circuit, with its many bonding wires, its highly reflective surfaces, and its intricate detail, presents a problem to photograph. Unless specially illuminated with supplementary diffuse light, the bonding wires reflect the light source and appear black in the photograph. The highly reflective surfaces tend to obliterate any fine detail present. The high contrast of the sample is likely to cause strong diffraction patterns. However, for very low power and with a simple microscope, these problems can be overcome by a method of illumination called axial lighting.

With ordinary lighting, the light source is directed on the specimen at approximately a 45-degree angle, giving a raw lighting. This usually causes too much contrast and, therefore, lack of detail. Axial lighting, however, is produced when the beam impinges on the specimen along the subject-lens axis, in the direction from the lens to subject. This arrangement is shown in Figure 31. Note that the light beam comes in at a 90-degree angle to the axis. It is then partially reflected to the subject by the mirror-like action of the 45-degree microscope cover glass. The rest of the light is transmitted through this glass and is lost. The photomicrograph is taken through the cover glass.

To photograph miniaturized electronic components at high magnification, where the objective and the specimen are very close, it becomes impossible to illuminate the specimen by direct lighting. Therefore, a vertical illuminator is needed. Such an illuminator, called an epi-condenser, has a special condenser built as a ring around the objective. The illuminating beam is projected to the specimen by this ring condenser. Several microscope manufacturers supply models in various designs. Also, a Lieberhuhn mirror can be used around a regular objective.

To enhance the effect of the axial lighting and to show all the detail possible in the bonding wires, an oblique, low-angle diffuse beam can be added. This will show texture in the specimen. The ratio of this lighting to the axial lighting is usually one diffuse to twice the quantity of axial lighting. However, this can change depending on the depth of the specimen.

Other methods of lighting that can be used are the vertical illuminator, which directs the illuminating beam down the optical axis and through the objective to the specimen, and the convergent cone of light from around the objective onto the specimen (e.g., Ultropak Illuminator manufactured by E. Leitz, Inc., 468 Park Avenue, New York, New York 10016). These methods give a low contrast lighting but render good definition without the problem of flare.

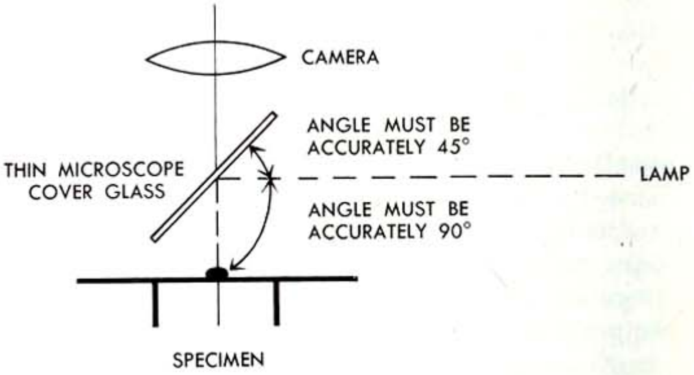

CAMERA
ANGLE MUST BE ACCURATELY 45°
THIN MICROSCOPE COVER GLASS
LAMP
ANGLE MUST BE ACCURATELY 90°
SPECIMEN

FIGURE 31—Elements for axial lighting. If a focused beam is used, the coil should be imaged in the plane of the camera lens.

Any lighting on the surface of the specimen tends to produce flare light. This degrades the image and reduces the contrast. Protecting the objective from any stray light, whether it is external or internal, will improve the contrast and image quality.

An excellent high-speed color film for this low level of lighting is KODAK High Speed EKTACHROME Film. All other materials of photomicrography apply and will not be repeated here.

Recording Replicas

In some applications, a thin plastic replication of a surface is mounted on a slide. This is valuable when the specimen is too opaque for brightfield photomicrography or when it is too bulky to place on the microscope. The transparent replica is illuminated with the condenser slightly off-center. Replication methods and materials and photomicrographic procedures are discussed in the literature on this highly specialized field.

GLOSSARY OF TERMS USED IN PHOTOMICROGRAPHY

Aberration—Any inherent deficiency of a lens or optical system which is responsible for imperfections in the shape or sharpness of the image as a result of failure to image a point or a straight line as such or an angle as an equal angle. The various forms of aberration can be reduced but not completely eliminated by assembling a lens from a number of different elements of compensating characteristics.

Achromat—A lens which brings light from two parts of the spectrum (strictly speaking two specified wavelengths) to the same focus. Modern achromatic lenses bring the blue and red regions of the spectrum to the same focus, thus reducing chromatic aberration.

Anisotropic—Exhibiting different properties when measured along different axes. A transparent, anisotropic material such as a crystal of calcite possesses different refractive indices in different directions through its mass (birefringence) and polarizes transmitted light.

Aperture—The lens opening through which light enters an optical instrument. The area of this opening is sometimes adjustable by an iris diaphragm or stop.

Apochromat—Lens which brings three chosen colors to the same focus. Apochromats used for microscope objectives are corrected for chromatic aberration for primary red, green, and blue light.

Bertrand Lens—Removable positive lens which can be fitted above the objective of a microscope to form an image of the objective's rear focal plane in the front focal plane of the eyepiece. It enables the *exit pupil* to be observed without removing the eyepiece when setting up Kohler illumination.

Birefringence—The refraction of light in two slightly different directions to form two rays. Polarizing filters that contain crystals oriented in one direction are birefringent.

Chromatic Aberration—Faults in the performance of lenses due to light of different colors coming to different planes of focus, the blue image being nearest the lens, or yielding images of different magnification.

Compensating Eyepiece—An eyepiece corrected primarily for use with apochromatic objectives, eliminating the color fringes found when ordinary eyepieces are used with such objectives.

Condenser—An optical assembly which concentrates light from a source into a beam. The beam is an evenly illuminated image of the source used in photomicrography, projection of slides, and printing negatives.

Contrast—The ratio of the amount of light transmitted or reflected by the most transparent and most opaque areas of an image.

Curvature of Field—A lens aberration in which images can be sharply focused only on a curved surface. Stopping down the lens reduces the visual effect by increasing the depth of focus.

Depth of Field—The region in front of and behind the focused distance within the subject in which object points still produce an image of acceptable sharpness.

Depth of Focus—Tolerance in the positioning of the image plane of a lens within which the lens forms an acceptably sharp image of an object at a given distance. In focusing, it represents, in effect, a focusing latitude.

Distortion—Aberration of a lens which causes the image to appear misshapen and deformed due to a gradual increase or decrease in magnification from the center to the edge of an image.

Empty Magnification—Magnification achieved by increasing the size of the image but not detail. This is caused by limited resolving power of the optical system.

f-Number—The ratio of the focal length of a lens to its effective aperture. A measure of the "speed" of a lens or its ability to gather light.

Interference Microscopy—Technique by which the illumination is divided into two beams, one of which passes through the transparent subject matter. The second beam is passed around, rather than through, the specimen. Recombination of the two beams results in interference patterns corresponding to the local thickness variations in the specimen.

Kohler Illumination—A method of brightfield illumination used in photomicrography and cinemicrography. A collector lens focuses an image of the light source on the condenser, which in turn focuses the field aperture in the plane of the specimen. This technique provides a uniformly illuminated field from nonuniform light sources such as coiled filament lamps.

Nanometer—SI unit of measure for electromagnetic radiation. Equals one meter times 10^{-9}. Visible light wavelengths range from 400 to 700 nanometers.

Numerical Aperture (N.A.)—A method of specifying the relative aperture of an objective lens and its resolving power. The numerical aperture value refers to the angle of the cone of light emitted by the condenser and accepted by the objective of the microscope. The formula is N.A. = N_D times the sine of U, where N_D is the refractive index of the objective and U is one-half the angular cone of illumination required to fill the aperture of the front lens of the objective. About 1,000 times the numerical aperture indicates the approximate limit of useful magnification of an objective.

Parfocal—The property of several lenses of different focal lengths of coming to focus at the same position.

Phase Contrast Microscopy—A technique for revealing the structural features of microscopic transparent objects whose varying but invisible differences in thickness result in varying differences in the phase of transmitted light. These phase differences are converted to visible intensity differences when part of the transmitted light has its optical path changed by about ¼ wavelength.

Polarizer—A transparent material which absorbs from light passing through it all vibrations except those in a single plane.

Polarizing Filter—A filter which transmits light polarized in one particular plane. Two such filters can be used to distinguish crystals of different types in photomicrography. If visually identical crystals differ in their birefringence, they will show differences in color and tone patterns if one polarizing filter is below and the other (which is capable of being rotated) is above the specimen.

Refraction—Change in direction of a ray of light passing from one transparent medium into another of a different optical density, that is, from air into glass. As the wavelength of the light shortens, the amount of refraction increases.

Refractive Index—The ratio of the speed of light in air to its speed in some other medium. This ratio determines how much light rays are bent.

Resolving Power—The ability of a lens to distinguish fine detail in the structure of a specimen. This ability is assessed by counting the number of closely-spaced objects or lines that can be recognized as separate in the final image. The formula is resolving power equals lambda divided by two times the numerical aperture, where lambda equals the wavelength of green light.

Spherical Aberration—A lens defect in which light rays passing through the outer regions of a lens converge and cross the lens axis nearer to the lens than rays passing through the central part of the lens. An object point at the lens axis is recorded as a disc of light.

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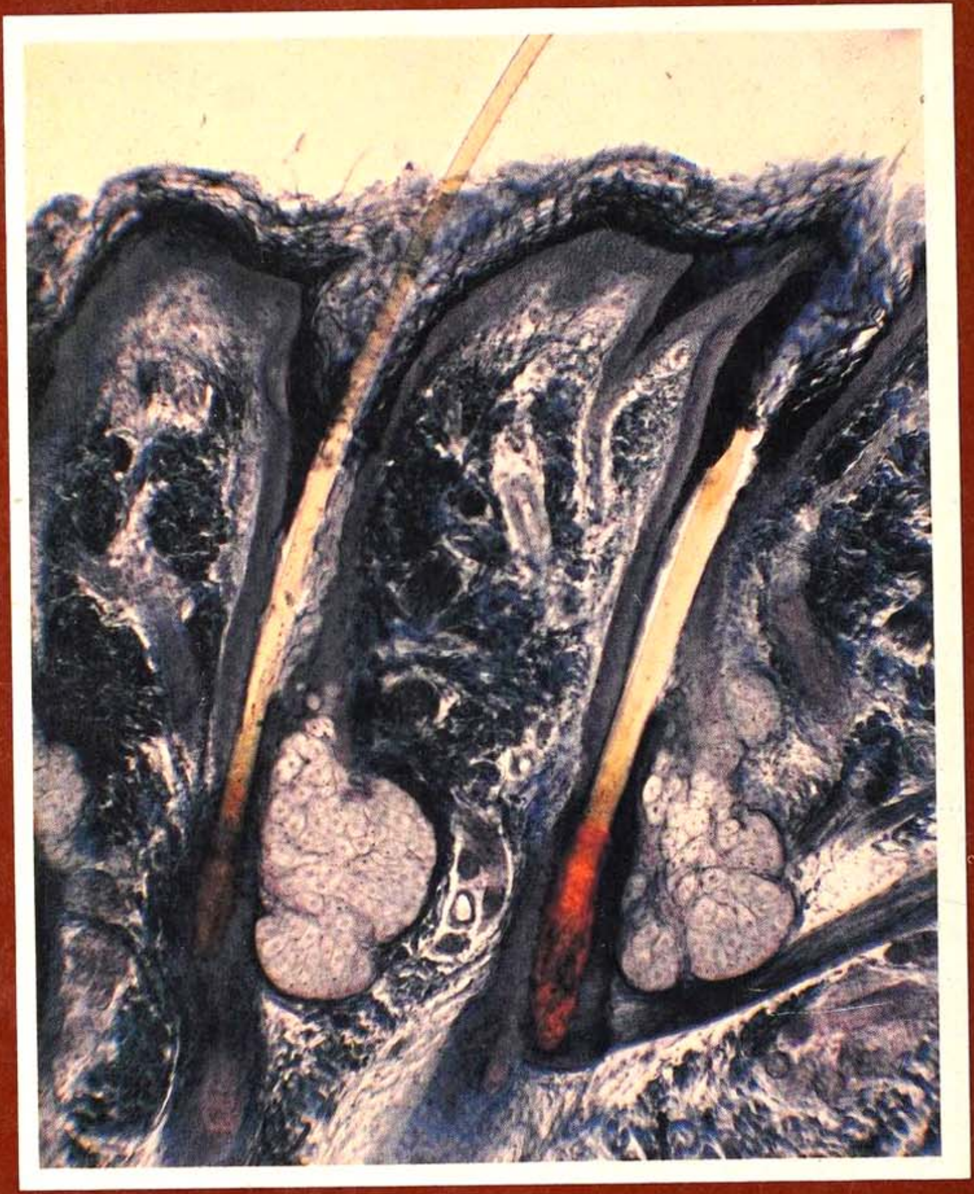
Other Kodak Publications of Interest to Readers of this Data Book

Eastman Kodak Company publishes more than 800 Data Books and technical pamphlets that provide authoritative, up-to-date technical information about Kodak products and their applications in many fields. These publications are listed in the *Index to Kodak Information,* Kodak Publication No. L-5, and can be obtained from your photo dealer, or ordered through the order form contained in the *Index*. You can request a complimentary copy of the *Index* by writing to Department 412-L.

Below are some of the publications listed in the *Index* that are of particular interest to scientific photographers:

KODAK Films for the Amateur (AF-1)
KODAK Filters for Scientific and Technical Use (B-3)
KODAK Color Films (E-77)
KODAK Black-and-White Films for Professional Use (F-5)
Processing Chemicals and Formulas (J-1)
Ultraviolet and Fluorescence Photography (M-27)
Applied Infrared Photography (M-28)
Medical Infrared Photography (N-1)
Photography of Gross Specimens (N-5)
Close-Up Photography, Volume I (N-12A)
Photomacrography, Volume II (N-12B)
KODAK Infrared Films (N-17)
KODAK Plates and Films for Scientific Photography (P-315)
Techniques of Microphotography (P-52)
Producing Slides and Filmstrips (S-8)
Photomicrography of Metals (P-39)



KODAK Photomicrography Color Film 2483

Recently, a new color film produced especially for the microscopist was introduced. It is called KODAK Photomicrography Color Film 2483. This film features high contrast, very high resolving power, excellent color saturation, and extremely fine grain. These features improve photographs of low-contrast specimens typically found in photomicrography. This film is available in 35 mm magazines and long rolls and in 4 x 5-inch sheets from your photographic dealer.

Shown here is a photomicrograph made with KODAK Photomicrography Color Film 2483 of a human scalp section (×105).

Kodak Publication No. P-2
Photography Through the Microscope
CAT 152 8371

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